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THE INTERPLAY BETWEEN MITOCHONDRIA-ENDOPLASMIC RETICULUM CONTACTS AND ALZHEIMER'S DISEASE

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Stockholm 2019

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Published by Karolinska Institutet.
Printed by E-Print AB 2019

ISBN 978-91-7831-389-1

The interplay between mitochondria-endoplasmic reticulum contacts and Alzheimer's disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

This thesis will be defended in Biomedicum 1, Biomedicum, Floor 3, Solnavägen 9, Solna Friday the 26th of April, 2019 at 9:30.

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「記憶を隠すことはできても、
歴史を消すことはできない。」

’You can hide memories, but you can't erase the history.’

in Colorless Tsukuru Tazaki and His Years of Pilgrimage by Haruki Murakami

ABSTRACT

Even though Alzheimer's disease (AD) was first described more than 100 years ago, we still have no treatment preventing the ongoing neurodegenerative process. Two major pathological hallmarks have been connected to AD: extracellular amyloid plaques (constituted by amyloid β-peptide – Aβ) and neurofibrillary tangles. Several biological processes have been shown to be altered in AD including mitochondrial functions, autophagosome formation and calcium (Ca^{2+}) homeostasis. Interestingly, all these processes have been shown to be regulated in mitochondria-endoplasmic reticulum contact sites (MERCS). Moreover, both the activity and the number of these contacts are affected in AD, which could explain the alterations of the biological processes mentioned above. However, it is still unknown if the alteration in MERCS causes the pathology or vice-versa. In this thesis, I have contributed to uncovering some of the mechanisms behind the interplay between MERCS and AD.

- In **Study I** we show that the number of MERCS is increased in brain biopsies of demented patients and that there is a reversed correlation between MERCS and Mini Mental State Examination (MMSE) scores. In the same study, we show that the number of MERCS positively correlate with aging and ventricular Aβ42 levels;
- In **Study II** we show that Aβ increases the number of MERCS in different models, leading to alteration in autophagosome formation and mitochondrial function;
- In **Study III** we show that the increase of MERCS, through acute knock-down of Mitofusin 2, leads to decreased levels of both Aβ40 and Aβ42 due to impaired γ-secretase assembly and activity;
- In **Study IV** we show that the translocase of the outer mitochondrial membrane (TOM) receptor protein TOM70 modulates Ca^{2+} shuttling from ER to mitochondria via IP3R3 at MERCS.

Altogether, these studies contributed to unravel the role of MERCS in AD. We show that MERCS dynamics changes throughout ageing and is accentuated in AD pathology, affecting several biological processes vital for overall cellular function. We believe this increase of MERCS could either trigger the neurodegenerative processes underlying AD or being an attempt to rescue neuronal dysfunctions. Moreover, MERCS modulation affects Aβ levels, which makes us believe that MERCS and Aβ regulate each other in a reciprocal manner.

RESUMO

Apesar da doença de Alzheimer (AD) ter sido primeiramente descrita há mais de 100 anos ainda não existem tratamentos que alterem o processo de neurodegeneração observado na patologia. Os dois principais marcadores moleculares associados com a doença são: placas amiloides no meio extracelular (constituídas maioritariamente pelo péptido β-amiloide - Aβ) e os novelos neurofibrilares no meio intracelular. Nos últimos anos, vários processos biológicos foram demonstrados estar alterados na AD incluindo função mitocondrial, metabolismo de fosfolípidos, formação de autofagossomas e homeostasia do cálcio (Ca^{2+}). Curiosamente, todos estes processos são regulados pelos locais de contacto entre a mitocôndria e o retículo endoplasmático (ER) (MERCS). Além disso, foi demonstrado que estes contactos estão aumentados na AD, o que pode explicar a alteração dos processes biológicos referidos acima. No entanto, ainda está por determinar se as alterações nos MERCS causam a patologia ou vice-versa. Esta tese contribui para o esclarecimento de alguns dos mecanismos por detrás da interação entre os MERCS e a AD.

- No **Estudo I** demonstrámos que o número de MERCS está aumentado em cérebros de pacientes com demência e que existe uma correlação inversa entre o número de MERCS e os resultados do Mini Exame do Estado Mental (MMSE). No mesmo estudo também demostrámos que o número de MERCS é directamente proporcional com a idade e os níveis ventriculares de Aβ42.
- No **Estudo II** demonstrámos que o Aβ aumenta o número de MERCS em vários modelos, levando a alterações na formação de autofagossomas e função mitocondrial;
- No **Estudo III** demonstrámos que o aumento de MERCS, através da redução dos níveis da protein Mitofusina 2, leva à diminuição dos níveis de Aβ40 e Aβ42 devido à alteração da conformação e actividade da γ-secretase.
- No **Estudo IV**, mostramos que o receptor TOM70 da translocase da membrana exterior mitocondrial (TOM) modula a transferência de Ca^{2+} do ER para a mitocôndria através da proteína IP3R3.

De modo geral, estes estudos ajudam a esclarecer o papel dos MERCS na AD. Nós demostramos que a dinâmica dos MERCS é alterada durante o envelhecimento e é mais acentuada na AD, afectando vários processos biológicos vitais para a célula. Portanto, acreditamos que o aumento dos MERCS pode resultar no processo de neurodegeneração observado na AD ou resgatar a disfunção neuronal. Além disso, a modulação dos MERCS afecta os níveis do Aβ, o que nos faz crer que os MERCS e o Aβ controlam-se mutuamente através de um mecanismo de *feedback*.

SAMMANFATTNING

Trots att Alzheimers sjukdom (AD) först beskrevs för över 100 år sedan har vi fortfarande ingen behandling som påverkar den pågående neurodegenerativa processen. Två viktiga patologiska kännetecken har kopplats till AD: extracellulära amyloida plack (utgörs av amyloid β-peptid-Aβ) och neurofibrillära nystan. Flera biologiska processer har visat sig förändras i AD inklusive mitokondriella funktioner, autofagosombildning och kalcium (Ca^{2+}) homeostasen. Intressant nog har alla dessa processer visat sig regleras i mitokondrie-endoplasmatiiska retikulets kontaktställen (MERCS). Dessutom har både aktiviteten och antalet av dessa kontakter visat sig öka i AD vilket skulle kunna förklara förändringarna av de biologiska processerna som nämnts ovan. Det är dock fortfarande okänt om förändringen i MERCS orsakar patologin eller vice versa. I denna avhandling har jag bidragit till att belysa några av mekanismerna bakom samspelet mellan MERCS och AD.

- I **Studie I** visar vi att antalet MERCS ökar i hjärnbiopsier från dementa patienter och att det finns en inverterad korrelation mellan antalet MERCS och MMSE-poäng. I samma studie visar vi att antalet MERCS positivt korrelerar med åldrande och ventrikulära Aβ42-nivåer.
- I **Studie II** visar vi att Aβ ökar antalet MERCS i olika typer av djurmodeller vilket leder till förändring i autofagosombildning och mitokondriefunktion;
- I **Studie III** visar vi att ökningen av antalet MERCS genom akut nedreglering av Mitofusin2 leder till minskade nivåer av både Aβ40 och Aβ42 på grund av nedsatt mognad och aktivitet av γ-sekretaskomplexet.
- I **Studie IV** visar vi att translokator av det yttre mitokondriella membranet (TOM) receptorn TOM70 modulerar Ca^{2+} transporten från ER till mitokondrier via IP3R3 vid MERCS.

Sammantaget har dessa studier bidragit till att förstå MERCS:s roll i AD. Vi visar att MERCS-dynamiken förändras genom åldrande och accentueras med AD-patologi, vilket påverkar flera biologiska processer som är viktiga för cellen. Vi tror att denna ökning av MERCS kan leda/bidra till den neurodegenerativa processen som pågår i AD eller rädda nervceller. Dessutom påverkar MERCS-moduleringen Aβ-nivåerna, vilket visar att MERCS och Aβ kontrollerar varandra på ett reciprokt sätt.

LIST OF SCIENTIFIC PAPERS

I. **Nuno Santos Leal**, Giacomo Dentoni, Bernadette Schreiner, Olli-Pekka Kämäräinen, Nelli Partanen, Sanna-Kaisa Herukka, Anne M Koivisto, Mikko Hiltunen, Tuomas Rauramaa, Ville Leinonen and Maria Ankarcrona
"Alterations in mitochondria-endoplasmic reticulum connectivity in human brain biopsies from idiopathic normal pressure hydrocephalus patients."
Acta Neuropathol Commun 6:102 (2018).

II. **Nuno Santos Leal**, Giacomo Dentoni, Bernadette Schreiner, Giovanni Meli, Gabriele Turacchio, Antonio Piras, Caroline Graff, Tamotsu Yoshimori, Maho Hamasaki, Per Nilsson and Maria Ankarcrona
"Amyloid β-peptide increases mitochondria-ER contacts and affects mitochondria function and autophagosome formation."
Manuscript.

III. **Nuno Santos Leal**, Bernadette Schreiner, Catarina Moreira Pinho, Riccardo Filadi, Birgitta Wiehager, Helena Karlström, Paola Pizzo and Maria Ankarcrona.
"Mitofusin-2 Knockdown Increases ER-Mitochondria Contact and Decreases Amyloid β-Peptide Production."
Journal of Cellular and Molecular Medicine 20 (9): 1686–95 (2016).

IV. Riccardo Filadi*, **Nuno Santos Leal***, Bernadette Schreiner*, Alice Rossi, Giacomo Dentoni, Catarina Moreira Pinho, Birgitta Wiehager, Domenico Cieri, Tito Calì, Paola Pizzo and Maria Ankarcrona
"TOM70 Sustains Cell Bioenergetics by Promoting IP3R3-Mediated ER to Mitochondria Ca^{2+}Transfer."
Current Biology 28 (3): 369–382.e6 (2018).
*These authors contributed equally to this work

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
AD	Alzheimer's disease
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CSF	Cerebrospinal fluid
Drp1	Dynamin-related protein 1
ER	Endoplasmic Reticulum
ETC	Electron transport chain
FAD	Familial AD
Grp75	Glucose-regulated protein 75
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IP3R3	Inositol 1,4,5-trisphosphate receptor type 3
LC3	Microtubule-associated protein 1A/1B-light chain 3
MAM	Mitochondria-associated ER membranes
MCU	Mitochondrial calcium uniporter
MERCS	Mitochondria-ER contact sites
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
MMSE	Mini-mental state examination
NFT	Neurofibrillary tangles
OMM	Outer mitochondrial membrane
Opa1	Optic atrophy 1

OXPHOS	Oxidative phosphorylation
p62	SQSTM1 / p62
PC	Phosphatidylcholine
PCN	Primary cortical neurons
PE	Phosphatidylethanolamine
PLA	Proximity ligation assay
PS1	Presenilin 1
PS2	Presenilin 2
PSer	Phosphatidylserine
PSs	Presenilins
SAD	Sporadic AD
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TOM20	Translocase of the outer membrane protein mitochondrial import receptor subunit TOM20
TOM70	Translocase of the outer membrane protein mitochondrial import receptor subunit TOM70
VDAC1	Voltage-dependent anionic channel 1
ΔΨm	Mitochondrial membrane potential

1 INTRODUCTION

1.1 ALZHEIMER'S DISEASE

"No matter how much suffering you went through, you never wanted to let go of those memories", Haruki Murakami wrote. For Alzheimer's disease (AD) patients, these memories unwillingly fade. Sadly, there is an awareness and a feeling of helplessness knowing that modern medicine still faces the same challenges as when, in 1901, Alois Alzheimer first described the disease.[1] Today, after more than one hundred years, even though diagnostic techniques have improved and the molecular mechanisms underlying the pathology are better understood, there are still no available drugs that prevent the progression of the disease. Why have therapeutics in AD been so challenging? Many scientists are still battling this question. This thesis further uncovers the role of mitochondria-ER contact sites (MERCS), one of several affected processes of the pathology, in the origin/progression of AD.

1.1.1 Definition and prevalence

According to the World Health Organization (WHO), dementia is defined as "a syndrome – usually of a chronic or progressive nature – in which there is deterioration in cognitive function (i.e. the ability to process thought) beyond what might be expected from normal ageing. It affects memory, thinking, orientation, comprehension, calculation, learning capacity, language, and judgement." (https://www.who.int) According to the WHO website "worldwide, around 50 million people have dementia, with nearly 60% living in low- and middle-income countries" and, since there are no drugs that affect the ongoing neurodegeneration, "the total number of people with dementia is projected to reach 82 million in 2030 and 152 in 2050." (https://www.who.int)

AD is an example of these dementias. In fact, AD is the most common form of these mental disorders in the world and it is associated with lower quality of life, considerable suffering for both patients and families/caregivers, and economic burden on society. Similarly to the WHO's definition of dementia, AD is described as a complex multifactorial neurodegenerative disease characterised by the decline of cognitive functions and loss of memory which can be associated with depression, confusion and aggressive behavior.[2] For AD, the numbers today are around 50 million cases worldwide but since this is a chronic ageing-related disorder and the

world population's life expectancy is increasing, the numbers are expected to augment to 1 in 85 people by 2050.[3–5]

1.1.2 Aetiology and pathophysiological hallmarks

Pathological hallmarks of AD include progressive loss of neurons (predominantly forebrain cholinergic neurons, and cortical and hippocampal glutamatergic neurons) and synapses. It also associated with accumulation of extracellular senile plaques [constituted of the amyloid β-peptide (Aβ)] in hippocampus and prefrontal, parietal and temporal cortices, and intracellular neurofibrillary tangles (NFT) (constituted of hyperphosphorylated tau protein) in hippocampus and entorhinal cortex.[6–10]

Even though there is no consensus on AD aetiology, the most accepted hypothesis to explain the origin of the neurodegenerative process in AD is the intra- and extracellular accumulation of Aβ in the brain, denominated amyloid hypothesis. In fact, for many years it was thought that the amyloid plaques were responsible for the neuronal death observed in the pathology; however new evidences shows that the plaque formation may actually be a way for cells to deposit toxic Aβ extracellularly.[11] Moreover, there is no correlation between the severity of cognitive impairment and plaque load. However, oligomeric forms of Aβ have been shown to be toxic both intracellularly and when applied extracellularly arising the idea that maybe these forms are responsible for the neuronal death.[12–14] Although in this thesis I will mainly focus on the amyloid hypothesis, I am aware that AD pathogenesis is complex and also involves other alterations like oxidative stress, mitochondrial dysfunction, synaptic loss, alteration in cholesterol metabolism and inflammation leading, ultimately, to neuronal death.[15,16]

1.1.3 Genetics and risk factors

Two different subtypes of AD have been described: sporadic AD (SAD) and familial AD (FAD).[7] While the exact aetiology of SAD is not known, FAD is caused by autosomal dominantly inherited mutations in the amyloid precursor protein (APP), presenilin 1 (PS1) or presenilin 2 (PS2). Although these mutations support the amyloid hypothesis (since they connect the pathology with the increased levels and aggregation of Aβ) they only account for 1% to 5% of the total AD cases. Moreover, FAD patients tend to have an earlier onset (from 30 years old) and more aggressive form of the disease when compared to SAD (with onset around 60 years old).[17–19]

Over 350 mutations have been found in APP, PS1 and PS2 and shown to be related to AD. (in https://www.alzforum.org/mutations) Relevant to this thesis are four mutations in the APP protein: London mutation (V717I), Swedish double mutation (KM670/671NL), Iberian mutation (I716F) and Artic mutation (E693G). All these mutations leads to an increase in the total Aβ levels except for the Artic mutation which increases the propensity of Aβ40 to aggregate, inducing the formation of protofibrils. (in https://www.alzforum.org/mutations)[20–22] Although, the mentioned autosomal genetic mutations explain the aetiology of FAD, the reason behind the accumulation of Aβ in SAD remains unknown. However, known risk factors have been shown to increase the risk of developing SAD, including age, alcohol, smoking, diet; physical, cognitive and social inactivity; and the presence a specific allele of apolipoprotein E (ApoE). [19,23] ApoE is a component of lipoproteins that traffic lipids through blood, including in and within the brain, and the most common occurring allele variants is ApoE3. Carriers of variant ApoE4 show a significantly increased risk of developing AD than carriers of ApoE3 (2-3 fold if in one allele and up to 12-fold if in both alleles).[24]

1.1.4 Disease progression, diagnosis and treatment

Despite the complexity of AD and the difference in onset, symptoms of both FAD and SAD are similar and include loss of short-term memory, decline of cognitive function (eg. language, decision making, abstract reasoning), incapability to perform at work and in social activities, changes in mood, and, ultimately, death commonly due to weakness, malnutrition and pneumonia.[18] While, in the past, AD was usually diagnosed in advanced stages of pathology, we now know that the pathology starts up to 20-30 years before the first clinical symptoms. The development of new and more sensitive biomarkers in combination with neurological exams, results in migration of diagnosis to pre-symptomatic patients. Today, diagnosis of AD is based on both neurological and cognitive tests (like the Mini-Mental State Examination – MMSE) as well as brain structure using magnetic resonance imaging (MRI) and biomarker assessment including the detection of different forms of Aβ and tau in cerebrospinal fluid (CSF) or by positron emission tomography (PET).[23,25,26] However, a definitive diagnosis of AD requires not only the clinical assessment but also a neurological examination of the *post-mortem* brain, to identify NFT and amyloid plaques.[18] Even though early dementia diagnoses have increased over the past years, there is a lack of preventive strategies and pharmacological treatments that

stop the ongoing neurodegenerative processes in AD. Therefore, there is a need to identify the molecular mechanisms underlying the pathology to allow for new and improved diagnostic and therapeutic strategies to be developed.[15] Treatment with acetylcholinesterase inhibitors and NMDA-agonist (memantine) results in slow down of the cognitive decline in patients with mild-to-moderate dementia, but does not modify the course of the illness.[18] Recently, the traditional concept "one target, one treatment" has been questioned. After failure of over 100 clinical monotherapy trials targeting Aβ, multi-target therapies that address various aspects of AD are thought to be the solution.[19]

1.1.5 Amyloid precursor protein and amyloid β-peptide

1.1.5.1 Amyloid precursor protein, cleavage products and Aβ generation

Aβ derives from the processing of amyloid precursor protein (APP).[27] The physiological role of APP and its cleavage products remain largely undetermined, however suggested functions are connected to neurite outgrowth, synaptogenesis, neuronal protein trafficking along the axon, transmembrane signal transduction, cell adhesion and calcium (Ca^{2+}) metabolism.[28] Moreover, while some APP fragments are thought to be neuroprotective (e.g. sAPPα), others are considered to be neurotoxic (e.g. Aβ42), suggesting that net effect of full-length APP and its metabolites on cellular activity may be a combination of these metabolites' functions, depending temporospatially on the proportion of levels of each APP metabolite.[29]

Although several publications have described different processes involved in APP trafficking and processing, the whole process is still not fully understood and there is no consensus in the scientific community about the exact mechanism. Inside the cell, APP is expressed at high levels, quickly metabolised and can undergo two different cleavage pathways: the non-amyloidogenic and the amyloidogenic.[4,30–32] In the first pathway APP is cleaved by α-secretase and γ-secretase, forming p3, soluble APPα fragment (sAPPα), amyloid precursor protein intracellular domain (AICD) and C83; in the second pathway APP is cleaved by β-secretase (BACE1) and γ-secretase forming Aβ, soluble APPβ fragment (sAPPβ), AICD and C99 (**Figure 1**). β-secretase is constituted of the β-site APP cleaving enzyme (BACE1) and it has optimal function in acidic pH (like at endosomes and lysosomes).[33] The γ-secretase complex is constituted of four different proteins: presenilin 1 (PS1) or 2 (PS2), anterior pharynx-defective 1 (APH-1), Nicastrin (NCT) and presenilin enhancer 2

(PEN-2).[34–37] The complex assembles and matures in the secretory pathway. APH-1 and NCT form the initial scaffolding complex, stabilising PS1 (or PS2). PEN-2 stabilises this complex by activating endoproteolysis of PS1 (or PS2).[34–37] Both full-length PS1 and PS2 undergo endoproteolysis in order to become proteolytically active, yielding the C-terminal fragment (CTF) and the N-terminal fragment (NTF) as by-products.[38]

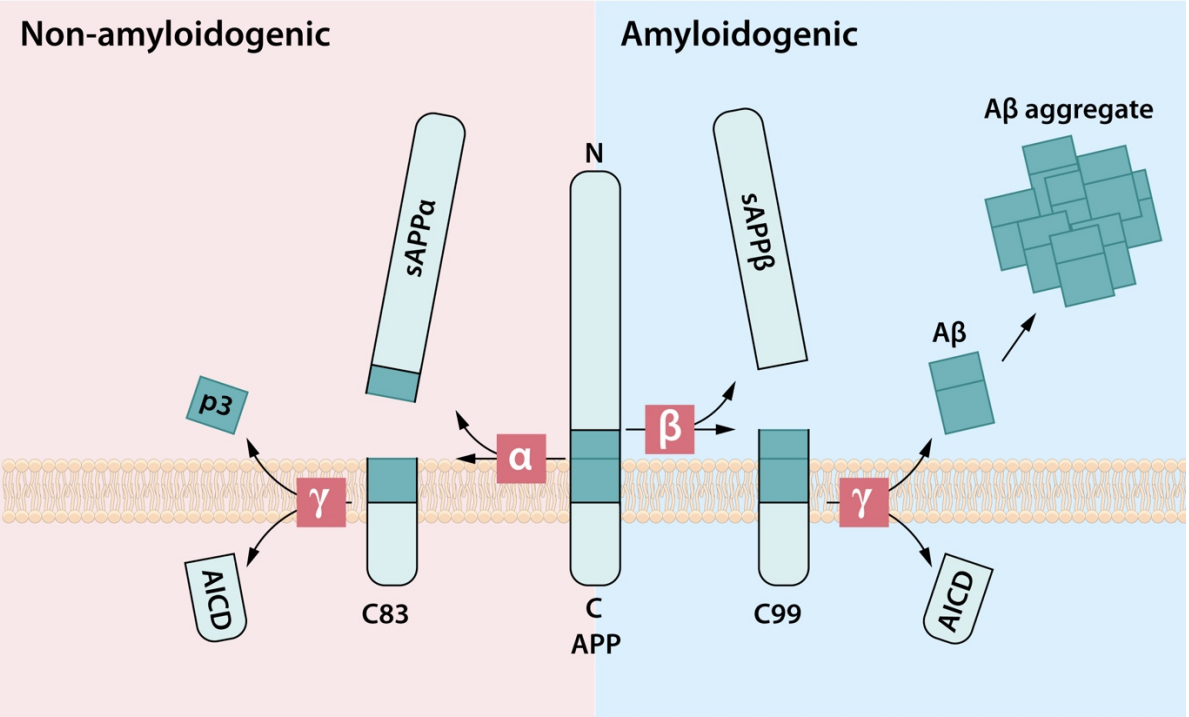

Figure 1. APP cleavage pathways. On the left the non-amyloidogenic pathway is shown where APP is first cleaved by α-secretase, forming C83 and sAPPα, and secondly by γ-secretase, forming p3 and AICD. On the right the amyloidogenic pathway where the first cleavage is performed by β-secretase, forming C99 and sAPPβ, and the second cleavage by γ-secretase, forming Aβ an AICD. Aβ can then form Aβ aggregates which will origin amyloid plaques observed in AD.

Due to the different locations of the various secretases, APP and its metabolites, as well as the optimal activity of β-secretase in an acidic environment, it has not been possible to describe where exactly the generation of Aβ occurs. This processing is a complex mechanism that involves several steps and organelles and where and when precisely they all converge to form Aβ remains unknown. Although, one hypothesis is that APP is synthetized in the endoplasmic reticulum (ER) and subsequently transported to the Golgi apparatus where it undergoes post-translation modifications, such as glycosylation, phosphorylation and sulfation.[39] In Golgi, APP can either be cleaved by α- or β-secretase.[33] If not cleaved in this organelle APP is transported to the plasma membrane (via the secretory pathway) where it can be

processed by α-secretase (which is enriched in the plasma membrane), forming the sAPPα and C83. APP that is not cleaved in the plasma membrane is endocytosed within minutes and recycled to the Golgi or to the lysosomes where it can be cleaved by β-secretase forming sAPPβ and C99. C99 is then delivered to the ER, via a currently unknown mechanism, where it is cleaved by the γ-secretase complex, producing two peptides, Aβ and AICD while the C83, derived from the non-amyloidogenic, forms the non-toxic p3 and AICD. In unaffected individuals, C99 is rapidly cleaved to Aβ40, which is ~ 40 amino acids (aa) in length. In AD, cleavage of C99 is shifted towards the aggregation prone Aβ42, which is ~ 42 aa in length, leading to the increased Aβ42:Aβ40 ratio, frequently observed in AD patients.[40]

Interestingly, even though β-secretase follows similar trafficking routes as APP, they are not present in the same vesicles being unable to interact.[41] These vesicles eventually interact when neuronal activity induces the convergence of these vesicles in recycling endosomes.[42,43] Similarly, γ-secretase has been detected in Golgi, endosomes and in other organelles like ER.[44,45] Therefore, the cellular distribution of APP determines which secretase cleaves it and which cleavage products are generated. While accumulation of APP in the plasma membrane results in non-amyloidogenic processing, internalization of APP into acidic compartments leads to the formation of Aβ through the amyloidogenic pathway. In the beginning of 2019, Liu and colleagues showed that β- and γ- secretase form a super complex that is responsible for the majority Aβ production. Interestingly, they also showed that BACE1 and PS1 co-localized, with a stronger overlap in the perinuclear region where mitochondria and ER are relatively abundant.[46–48] Recent studies report that β-secretase, γ-secretase and Aβ production occur in intracellular lipid rafts at mitochondria-ER contact sites (MERCS).[52–55] This will be further developed in subheading **1.5.1 - β-secretase, γ-secretase, Aβ formation and MERCS.** Previously, PS, APP, β- and γ-secretase have been reported to be present in lipid rafts of the PM, although the activity of γ-secretase here is low.[49–51]

1.1.5.2 Aβ function and metabolism

As mentioned before the two main forms of Aβ involved in AD are Aβ40 and Aβ42, the last one being more toxic and prone to aggregation. The aggregation of Aβ is determined not only by the relative proportion of Aβ species but also by their concentration, pH, temperature and ionic strength of the solution.[15] Although Aβ seems to affect mostly neurons, this peptide is also produced in astrocytes, microglia and other organs besides the brain such as the kidney, heart and liver.[15] Since Aβ42 is more common in the brain while Aβ40 is more dominant in the peripheral tissues, this could explain why accumulation of Aβ is increased in the brain as compared to other peripheral tissues.[15]

The formation of Aβ via subsequent cleavage by β- and γ-secretase is widely accepted for neurons, however the mechanisms responsible for Aβ clearance are not fully elucidated. Intracellularly, Aβ can be cleaved either by insulin degrading enzyme (IDE) in the cytosol and endosomes or by the presequence peptidase (PreP) in the mitochondrial matrix. If not degraded intracellularly, Aβ is secreted extracellularly by synaptic vesicles, exosomes, and, surprisingly, autophagosomes.[56–58] Extracellularly, Aβ can either be degraded by neprilysin at the plasma membrane or taken up by glia cells and macrophages. Moreover, there is also an efflux of Aβ into the peripheral circulation [through the blood brain barrier (BBB) and CSF].[15,59,60] In the periphery, Aβ can be catabolised by leukocytes and hepatocytes, excreted via bile or urine or cleared by Aβ-binding proteins (eg. albumin, ApoE).[15,61] Notably, it is discussed that whilst Aβ40 is mainly degraded intracellularly, Aβ42 is degraded extracellularly. Therefore, it is believed that failure of Aβ clearance (especially Aβ42) is an important cause of sporadic AD.[62]

As described above Aβ is believed to be one of the causes of AD, however non-pathological functions have also been associated with Aβ such as: antioxidant and antimicrobial activity, activation of signalling proteins, modulation of cholesterol transport, transcription factor and kinase activation. [19,63,64] Aβ was also shown to regulate neuronal homeostasis (in picomolar concentrations) through stimulation of post-tetanic potentiation, long-term potentiation and presynaptic transmitter release.[65]

1.1.6 Tau protein

The other major molecular hallmark of AD are NFT formed by the intracellular accumulation of hyperphosphorylated tau protein. Tau was first described in the 1970s during a study involving microtubules, as one of the major components of the neuronal cytoskeleton. Nowadays, it is known that the main role of this protein is to assemble tubulin (the “building block” of the microtubules) into microtubules, regulating their stability. Microtubules are essential for neuronal homeostasis and function since they contribute to the their structural support and the normal morphology of the neurons and are involved in intracellular trafficking and cell division.[66,67] The binding of tau to the microtubules is regulated by kinases and phosphatases and, it has been postulated, that in AD there is an up-regulation of tau phosphorylation (hyperphosphorylation) preventing tau from binding microtubules which could lead to disorganisation and collapse of the microtubule network.[68,69] This hyperphosphorylation of tau is also believed to cause tau polymerisation and formation of fibrillary structures leading to generation of NFT.[70,71] NFT are believed to cause cell dysfunction (eg. decrease in glucose, lipid metabolism, ATP synthesis and others) due to the alteration in the organelle distribution within the cell.[72]

1.2 MITOCHONDRIA

Several organelles and biological processes have been shown to be affected in AD. One example of these affected organelles are mitochondria. If we imagine a cell as a city, mitochondria would be its powerhouse since they are the main source of energy in the cell, under the form of adenosine triphosphate (ATP). While primitive prokaryotic cells lived without mitochondria for around two billion years, eukaryotic cells are dependent on this organelle for cellular respiration and ATP production.

1.2.1 Mitochondrial structure

Structurally, mitochondria are constituted by two lipid bilayer membranes: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The compartment between the two membranes is called mitochondrial intermembrane space (IMS) whereas the innermost compartment is referred to as mitochondrial matrix **(Figure 2).** The structure and composition of each compartment defines its function.[73]

The OMM, as the name suggests, is the outer membrane that encloses the organelle and gives the shape and morphology to mitochondria. It contains a high number of integral membrane proteins called porins which allow molecules and small proteins up to 5000 daltons to diffuse through the membrane, while larger proteins cross this membrane via the translocase of the outer membrane (TOM) complex.[73] Furthermore, OMM also contains other enzymes responsible for the elongation of fatty acids, oxidation of epinephrine and degradation of tryptophan.[74] OMM has also an important role in cell death since its disruption leads to the leakage of IMS proteins, inducing cell failure and death.[74]

The IMM is particularly impermeable to most metabolites and ions due to its phospholipid composition. Cardiolipin, the major phospholipid of IMM, is constituted of four fatty acid chains instead of the typical two. This feature is extremely important for mitochondrial function because it allows the generation of mitochondrial membrane potential ($\Delta\Psi m$) during cellular respiration. Invaginations of the IMM, called cristae, increase its surface area and it is where the complexes of the electron transport chains (ETC).[73,75–77] These cristae can be remodelled according to the metabolic requirements of the cell and recently they have been connected to programmed cell death as they can release pro-apoptotic factors.[78]

The IMS is the aqueous compartment in between the OMM and the IMM. The IMS regulates protein import from the OMM to the IMM or matrix and protons

pumped across the IMM by ETC activity are stored in the IMS until travelling back to the matrix, whilst powering ATP generation through the F_OF_1-ATP synthase.[73,79]

Lastly, the matrix is the other aqueous compartment of mitochondria and it harbours mitochondrial DNA (mtDNA) and the proteins encoded by mtDNA are transcribed and translated by mitochondrial ribosomes. Furthermore, it is also in the matrix where the series of chemical reactions in the tricarboxylic acid (TCA) cycle occurs and where the reduced nicotinamide adenine dinucleotide (NADH) is formed as a by-product from the TCA cycle.

1.2.2 Mitochondrial DNA

Structurally, mtDNA is a circular double stranded DNA constituted of around 17.000 base pairs, encoding 11 messenger RNAs (which give origin to 13 proteins), 2 ribosomal RNAs (12S and 16S) and 22 transfer RNAs. Interestingly, the 13 proteins with mtDNA origin all belong to complexes of the respiratory chain. Since the ETC complexes are comprised of 92 proteins, mitochondria-encoded proteins account for only a very small fraction whilst the majority of these are nuclear encoded. In addition, nuclear DNA encodes a further 35 proteins that are required for the assembly of the respiratory chain even though they are not part of the mature complexes themselves.[80,81] The reason why not all mitochondrial proteins are nuclear encoded is not known, but it is believed to be related with regulatory features of mitochondrial functions (eg. mitochondrial membrane potential or redox status) that require the presence of mtDNA close to the respiratory chain.[81,82] In humans, it has been thought for a long time that mtDNA has an exclusively maternal origin. However, rcently it was shown for the first time that in some cases paternal mtDNA can be passed on to the progeny.[83]

1.2.3 Mitochondrial protein import and TOM machinery

While mtDNA encoded proteins are transcribed and translated in the mitochondrial matrix, the majority of mitochondrial proteins are encoded in the nucleus, translated in the cytosol and transported into mitochondria.[80,81] In order for mitochondrial proteins to be imported into mitochondria they require specific import signals in their polypeptide sequence. The most frequent mitochondrial import signal is a presequence that exists at the N-terminal end of the precursor protein. After being imported, this presequence is cleaved off to allow proper folding of the protein.[84,85] In the matrix, this now free presequence can penetrate IMM and disrupt

mitochondrial ΔΨm, due to its biochemical nature. Therefore, they are further degraded by proteases such as presequence protease.[86]

Upon reaching mitochondria, the precursor protein faces the first import machinery, the translocase of the outer membrane (TOM) complex. TOM complex is constituted by the receptors TOM20 and TOM70/71, the pore of the complex TOM40, TOM22 and the small assistance TOM proteins (TOM5, TOM6 and TOM7).[87] The main function of TOM20 and TOM70/71 is to identify if the precursor proteins have the proper targeting signal (presequence) and serve as the initial docking and checkpoint station. These proteins have been described to perform similar functions but to bind to different subsets of precursor proteins. While TOM20 mainly binds to the hydrophobic part of the N-terminal presequences, TOM70 and TOM71 mainly recognises the hydrophobic internal targeting signals.[88–90] Upon recognition by TOM20 or TOM70/71, the precursor protein binds to TOM22 and TOM5 which allows its passage through TOM40, the central aqueous pore of the complex.[91,92] Precursor proteins then cross IMS until they reach the translocase of the inner membrane 23 (TIM23) complex. The hydrophobic signals in the presequence will determine if a protein will permanence in the IMM by a stop transfer mechanism or if it gets translocated to the matrix.[93,94] Unlike the import through the OMM, the transport from the TIM23 complex into the IMM or matrix is ΔΨm-driven. Depending on the type of presequence, TIM23 mediates the translocation of the precursor protein into the matrix or the integration into the IMM. Proteins translocated into the mitochondrial matrix have to go through a final machinery that is ATP-driven, the translocase-associated import motor (PAM). Curiously, IMS protein selection is performed in a different way. IMS proteins usually have a high-content of cysteines so, as they emerge from the TOM40 pore, they form disulfide bridges due to the oxidative environment, “arresting” the protein in the IMS **(Figure 2)**.[95]

1.2.4 Glycolysis and oxidative phosphorylation: ATP production

ATP is produced mainly through two differences processes: glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). In the majority of human cells, glucose undergoes glycolysis where it is partly degraded, producing pyruvate. Per cycle, glycolysis produces only a small amount of ATP and NADH when compared to OXPHOS. Pyruvate can then travel inside mitochondria where it interacts with coenzyme A (CoA) forming acetyl-CoA. Next, this molecule enters the tricarboxylic acid (TCA) cycle, producing a large amount of NADH and flavin adenine dinucleotide

($FADH_2$). Electrons prevenient from NADH and $FADH_2$ are then passed along the ETC where the majority of ATP is produced by OXPHOS.[73]

The ETC is constituted of four redox complexes present in the IMM: Complex I (NADH:ubiquinone oxidoreductase/NADH dehydrogenase), Complex II (Succinate: ubiquinone oxidoreductase/Succinate dehydrogenase), Complex III (Ubiquinol:Cytochrome *c* reductase), Complex IV (Cytochrome *c* oxidase). These complexes are responsible for pumping protons from the matrix into the IMS (except complex II), using the flow of electrons to drive this process. Through a reduction-oxidation reaction, NADH (electron donor) transfers an electron to the Complex I (electron acceptor) while $FADH_2$ (electron donor) transfers to the Complex II (electron acceptor). Then, through the electron carrier Ubiquinone (Q) transfer electrons to complex III and through cytochrome c they are transferred to Complex IV. In Complex IV, oxygen is the last acceptor of electrons and H_2O is formed with this electron and protons from the matrix.[73] The transfer of electrons through the different ETC complexes occurs due to consecutive increase of electronegativity of the acceptors. With the pumping of the protons, an electrochemical gradient (responsible for the creation of $\Delta\Psi m$) is established due to the differences in charges and concentration of protons between IMS and matrix. Due to the impermeability of the IMM, protons cannot pass through this membrane so they travel back to the matrix through the channel of the complex V (F_OF_1-ATP synthase) inducing the rotation in the complex which induces phosphorylation of ADP and inorganic phosphate into ATP **(Figure 2)**.[73,96]

1.2.4.1 The Warburg effect

Otto Warburg was the first to describe that cancer cells tended to perform aerobic glycolysis, even in the presence of oxygen and functional mitochondria, producing high amounts of lactate. Warburg realised that in order to prevent tumour grow he had to inhibit both glycolysis and OXPHOS (by removing glucose and oxygen) since the removal of just one of them was not enough.[97] Nowadays it is known that the Warburg effect is associated not only with tumours but also with other proliferating or developing cells, including immortalised cell lines used commonly worldwide. These cells have shown an increased uptake of glucose and produce, like the tumours, high levels of lactate even in the presence of oxygen and fully functional mitochondria.[98] It is important to keep in mind that several of the studies mentioned in this thesis do not take this effect into account, meaning that changes

in mitochondrial metabolism could be "masked" due to "understimulated" mitochondria. Curiously, neurons rely on OXPHOS to meet their energy demands and therefore it is believed that the Warburg effect should not be overly pronounced in these cells. In fact, astrocytes provide extra lactate to neurons.[99]

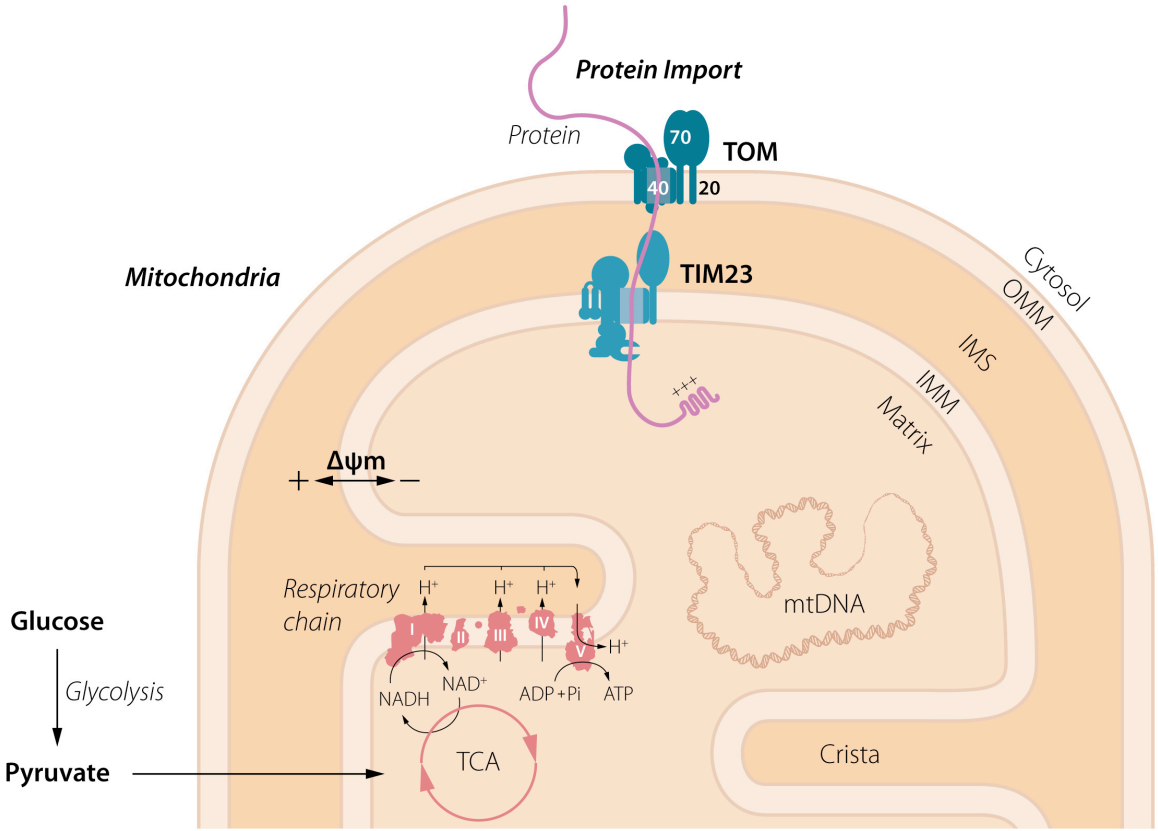

Figure 2. Overview of mitochondrial functions relevant for this thesis. Mitochondrial protein import mechanism occurs via TOM and TIM23 complexes and energy production (in the form of ATP) formed through glycolysis, TCA cycle and oxidative phosphorylation in the respiratory chain.

1.2.5 Mitochondrial dynamics: fusion and fission

The mitochondrion is frequently represented as a static rod-like isolated organelle but, in fact, this organelle is extremely dynamic and forms an extensive network that extends throughout the cell. However, mitochondrial morphology can be very different according to particular cell types and tissues since this morphology is highly linked to the organelle function, metabolic demands and health state. This morphology of the mitochondria is controlled through dynamic cycles of fusion and fission **(Figure 3)**.[77,100–102] These alternations between fusion and fission protect the mitochondria from excessive Ca^{2+} levels, mutant mtDNA and oxidative damage, all of which can influence mitochondria function. However, during ageing and in

neurodegenerative disorders, an unbalance in mitochondrial dynamics is frequently observed, leading to an increase of fragmented mitochondria.[103,104]

1.2.5.1 Fusion

Several proteins have been reported to be involved in mitochondrial fusion among which mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) and optic atrophy 1 (Opa1) are the most studied. While Mfn1 and Mfn2 are responsible for the fusion of the OMM, Opa1 is responsible for the fusion of the IMM. Mfn1 and Mfn2 present similar structures, compromising around 750 amino acids and containing a GTPase domain in their N-terminal region, while the C-terminal region is anchored to the OMM by transmembrane domains. Even though these two proteins are structurally identical, Mfn1 is thought to be crucial for mitochondrial docking and fusion while Mfn2 has a lower GTPase activity and it is thought to stabilise the interactions between mitochondria.[105,106] For the fusion event, both proteins form homo- or heterodimers through their HR2 domains **(Figure 3)**. Ablation of either *Mfn1* or *Mfn2* in mice is embryonically lethal and impairment of Mitofusins GTPase activity prevents mitochondrial fusion.[107,108]

Opa1 is anchored to the IMM by the N-terminal transmembrane domains while the GTP-binding and GTPase effector domain are the C-terminal region facing the IMS **(Figure 3)**. Opa1 exists in different isoforms due to alternative splicing and proteolytic cleavages. While the long form (L-Opa1) is involved in the fusion of the IMM, excessive accumulation of the short form (S-Opa1) can lead to mitochondrial fragmentation. S-Opa1 is a short soluble form (found in the IMS), derived from the cleavage of L-Opa1 by OMA1 and YE1L and is also involved in controlling cristae shape, together with mitochondrial contact site and cristae organizing system (MICOS).[109,110] Genetic ablation of *Opa1* leads to impairment of mitochondrial fusion, originating fragmented mitochondria. As for Mitofusins, Opa1-ablated embryos are not viable.

1.2.5.2 Fission

In addition, as in the fusion process, several proteins have been reported to be involved in mitochondrial fission but in this thesis, I will focus on dynamin-related protein 1 (Drp1). Upon stimuli, this cytosolic protein migrates to the OMM where it oligomerizes in a ring-like structure. As the fusion proteins mentioned before, Drp1 is also a GTPase, and upon GTP binding a conformational change occurs in the protein, leading to the strangling of the OMM by the ring-like structure, causing

fission. Several proteins have been reported to recruit Drp1 to the OMM including Fis1 and Mff **(Figure 3)**. Drp1 activity can be modulated by these receptors/adaptor proteins by, for example, influencing its GTPase activity.[111] Thus, this allows the possibility of regulating Drp1 in response to different cellular energy states. Mice with neuronal Drp1 KO die after birth, showing increased mitochondrial fusion, abnormal mitochondrial distribution along the axon, reduced neurites and impaired synapse formation.[112]

Albeit fission and fusion of mitochondria are extremely important in all cell types, they are essential for neuronal development, survival and function due to the high energy demand of these cells.[113] Due to the morphology of neuronal cells, fusion and fission are crucial for mitochondrial proliferation and distribution in the entire cell, even in the longest axons and dendrites. In neurons, mitochondrial fusion facilitates mitochondrial movement across long distances while mitochondrial fission allows mitochondrial renewal, redistribution and proliferation into synapses and post-synaptic terminals.[103,114,115]

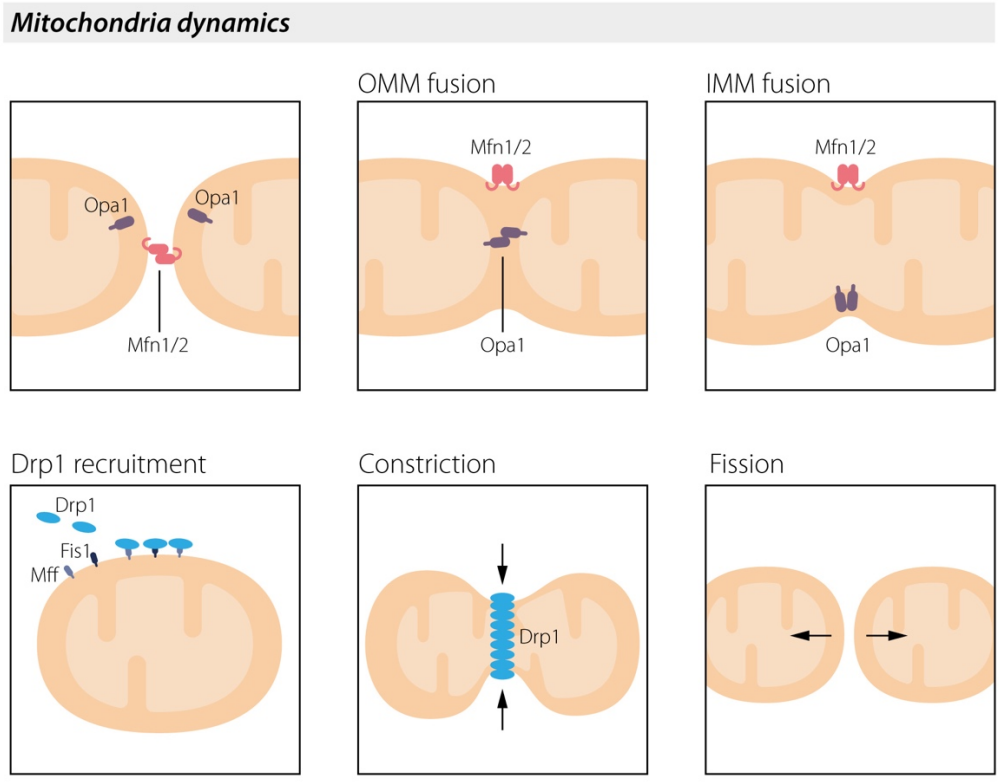

Figure 3. Representation of mitochondrial fusion and fission. Mitofusin 1 and 2 (Mfn1 and Mfn2) are involved in the OMM fusion and Opa1 in the IMM fusion. Drp1 is recruited to mitochondria by, for example, Fis1 and Mff which will lead to the constriction of mitochondria, leading to its fission.

1.2.6 Mitochondria quality control and mitophagy

Under certain conditions, when mitochondria stop working properly and its ΔΨm is lost, this could lead to consequences for the whole cell, culminating in induction of apoptosis and cell death. Therefore, there are several mechanisms that work as mitochondrial quality control. Those include the previously described mitochondrial dynamics (fusion and fission) where mitochondrial errors are diluted within the mitochondrial network or repaired.[103,116,117] When irreversibly damaged, impaired mitochondria are first separated from the rest of the network by fission and permanently eliminated by mitophagy.[101] Mitophagy is a selective type of autophagy (further developed in the subheading **1.3.3.5 Autophagosome formation**) where mitochondria are engulfed into a vesicle enriched in hydrolases (autophagolysosome), leading to the degradation of the organelle. Several mechanisms have been described to trigger mitophagy such as PINK1/parkin dependent mitophagy. In normal conditions PTEN-induced putative protein kinase 1 (PINK1) is imported into mitochondria via TOM and TIM23 complexes. Upon integration in the IMM, PINK1 is cleaved by the presenilin-associated rhomboid-like protein (PARL).[118] If the ΔΨm is disrupted, PINK1 is not imported into the IMM and PARL cannot cleave PINK1. In this case, PINK1 accumulates in the OMM, leading to the recruitment of Parkin. Parkin's E3 ubiquitin ligase activity is activated, modifying cytosolic and OMM proteins by adding poly-ubiquitin to them (eg. Voltage-Dependent Anionic Channel 1 (VDAC1), TOM40, TOM70, Mfn1 and Mfn2).[119–122] These poly-ubiquitinated proteins are then found and recognised by p62/SQSTM1 (p62) leading to the recruitment of the autophagy-related proteins and formation of the autophagosome (further developed in the subheading **1.3.3.5 Autophagosome formation**).

1.2.7 Dysregulated mitochondrial function/structure and pathology

Due to their pivotal role in regulating key cellular events, alterations in mitochondrial structure or function can have a major impact on normal cell homeostasis, leading to different disorders in humans. In fact, during ageing, mitochondria progressively becomes damaged and dysfunctional, leading to alterations in reactive oxygen species (ROS) production, protein folding and ATP production, and eventually cell death.[123] These alterations can then be associated with disorders like cancer and diabetes mellitus type 2.[102,124] Other mitochondrial disorders are associated with mutations in mtDNA (eg. myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and lactic acidosis).[102,125] Mutations in proteins regulating mitochondrial dynamics also lead to neuropathies like Charcot-Marie-Tooth disease type 2A (mutation in Mfn2) and autosomal dominant optic atrophy (mutation in Opa1).[126–129]

Considering their major role as energy producers, it is comprehensible that the most affected tissues upon mitochondrial dysregulation are the ones with the highest energy demand like the muscle, brain and heart.[77,130] In fact, it is quite surprising that while the human brain weighs only 2% of the total body weight it consumes 20% of all the oxygen and glucose during a resting state.[131] Nowadays, it is believed that the reason for this outstanding number is related to the fact that neurons rely mostly on complete oxidation of glucose via OXPHOS to produce energy (they are unable to switch to glycolysis if OXPHOS becomes limited) and the fact that they are unable to store glycogen (storage form of glucose).[132] Therefore, mitochondria are extremely important to neurons due to their high energy demand which includes: maintaining $\Delta\Psi m$, axonal and dendritic transport (that can be up to one meter in certain neurons), and the release and uptake of neurotransmitters.[103,133] Furthermore, due to the polarised morphology of neurons, mitochondrial distribution to axons and dendrites is essential for cell survival.[103,134]

Mitochondria have also been extensively linked to AD and this relationship will be further developed in the subheading **1.5.2 Mitochondria, ER and MERCS dysfunction in Alzheimer's disease.**

1.3 ORGANELLE CONTACT SITES: FOCUS ON MITOCHONDRIA AND ENDOPLASMIC RETICULUM JUXTAPOSITION

Organelles in eukaryotic cells were for many years thought to be well-defined membrane-enclosed components and separate entities. Historically, the majority of these organelle structures and functions were characterised, originating the impression that each organelle is a separate entity and exert a clear role for cell homeostasis. In 1977, upon the discovery of the clathrin-coated vesicles, cell biologists started realising that organelles communicate among themselves.[135–137] For example, the interplay between at least five organelles has been described to be needed for the synthesis of cholesterol: ER, Golgi apparatus, plasma membrane, mitochondria and nucleus.[138] Nowadays, it is known that organelles form highly organised networks and that this communication between organelles is essential for organelle development and function. This inter-organelle communication can occur through different mechanisms, for example either through vesicles or through organelle contact sites. Organelle contact sites is a fairly new area in research but this field has been developing rapidly and several associated processes and mechanisms have already been revealed. In this thesis, I will focus on the interaction between ER and mitochondria.

1.3.1 Mitochondria-ER contact sites and mitochondria-associated ER membranes

Mitochondria-ER contact sites (MERCS) were first observed in 1952 in rat liver and the first mitochondria-associated ER membranes (MAM) isolations from subcellular fractionations were performed in 1958, 1963 and 1971.[139–143] During this period, it was often interpreted that this juxtaposition was either an artefact of sample preparations or contaminations. In 1977, when Shore and Tata discovered that ER could be divided into two different fractions [rough ER (RER) and smooth ER (SER)] they, and other researchers, realised that mitochondria were attached differently to these different types of ER, being strongly connected with SER.[144–146]

ER is one of the largest organelles in the cell, sometimes occupying half of the total membrane area of a cell. The principal functions of ER are the biosynthesis of proteins (in RER) and lipids (in SER).[73,138] The rough appearance of RER is due to the attachment of ribosomes to the ER, which allows co-translation of transmembrane proteins. This process prevents protein misfolding by avoiding their release into the cytosol and exposing their hydrophobic content. It has been reported

that RER contains over 20 proteins that are absent in the SER, which are thought to help ribosomes bind to the ER.[73] SER is not as common as RER and ultra-structurally is characterised for not being associated with ribosomes. Unlike RER, SER possesses sites where transport vesicles carrying synthetised proteins and lipids bud off, called ER exit sites. These vesicles are then transported to other organelles (eg. Golgi apparatus) so the proteins can be further matured. Other SER functions include synthesis of steroid hormones from cholesterol, lipoproteins particles and phospholipids (phosphatidylcholine), and storage of Ca^{2+}. The release and uptake of Ca^{2+} from the ER are involved in several rapid responses within the cell. This sequestration is possible due to the high concentration of Ca^{2+}-binding proteins [eg. sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)] that pumps Ca^{2+} into the lumen at cost of ATP.[73]

However, it was not until 1990 that MAM was first described and a specific function attributed to this cell domain. Indeed, Jean Vance showed that phosphatidylserine (PSer), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are synthetised in MAM, in the presence of mitochondria.[147] Even though the terms MERCS and MAM have been used interchangeably but they do not represent the same thing. Therefore, I would like to point out some of the differences in the terminology. MERCS is often used when referring to the both OMM and ER, including the architecture and ultrastructural organisation of the contact. MERCS can be visualised by EM and is the physical platform where the processes occur. On the other hand, MAM has been given two meanings:

- The biochemically distinct region of the ER that behaves like a lipid-raft domain and is in contact with OMM;[138,148–150]
- The enriched ER and mitochondrial membranes fraction derived from subcellular fractionation. In other words, the biochemical essence of the MERCS.[151,152]

In this thesis, I will refer to MAM as Jean Vance did when she first introduced the term in 1994, therefore referring to the lipid-raft domain of the ER. MAM is a specialised region of the ER membrane with a lipid raft composition allowing it to interact with the OMM. Even though it was previously believed that mitochondria mainly interact with SER we now know that both SER and RER form MERCS with the OMM. In fact, in both cases, the organelles can run parallel to each other for dozens of nanometres, always separated by a cleft. The amount of ER in contact

with mitochondria is highly dependent on the needs of the cell and its metabolic state.[153] In HeLa cells, early phases of ER stress induce an increase of more than two-fold in the number of MERCS, while the exposure of RBL-2H3 cells to apoptotic stimuli decreased the average distance between ER and mitochondria from 28.2 nm to 20 nm.[154,155] Another example was shown by Sood and colleagues when, upon loss of thr mTORC1 nutrient-sensing pathway, there was an increase of the proximity between ER and mitochondria from 14 nm to 20 nm and an increase of average length of contact from 145 nm to 270 nm, while the number of MERCS did not change.[153] It is believed that the MERCS profile could be a distinct structure signature of different cell types and for each different metabolic state. For example while MERCS are typically absent in synapses of neurons of the dentate gyrus, in hepatocytes 25% of mitochondria have at least one MERCS and in HeLa cells this ranges between 5% and 20%.[151,153,156]

Curiously, not many studies have made detailed analyses of the structure, number and length of MERCS, and even less studies have considered the distance (cleft) between ER and mitochondria. The general consensus is that an increase of the number and length of MERCS correlates with increased MERCS function, however it is still highly debated what may happen when the distance between ER and mitochondria increases or decreases. By transmission electron microscopy (TEM), Csordas and colleagues identified that the average distance for this cleft is ≈ 10nm between SER and OMM and ≈ 25 nm between RER and OMM. After that, several publications have detected different distances between ER and mitochondria from 10 to 80 nm, distinguishing the contacts between close contacts (<30 nm) and long-distance contacts (>30 nm) **(Figure 4)**.[53,151,157] Recently, Giacomello and Pellegrini suggested that different distances between ER and mitochondria are related with different “types” of MERCS (with different functions) where the distances between the two organelles are regulated by different tethering and functional proteins (eg. from 15 nm for Ca^{2+} exchange between ER and mitochondria up to 50 nm to accommodate the autophagosome biogenesis).[151]

This juxtaposition between the ER and mitochondria is known to be transient and does not require fusion or overlap of membranes since crude mitochondria fraction treated with high salt concentration, detergents and short proteolysis disrupted these connections.[151,154,158] Therefore, we now know that MERCS formation requires proteins since proteinases can cause separation of the organelles.[154,159]

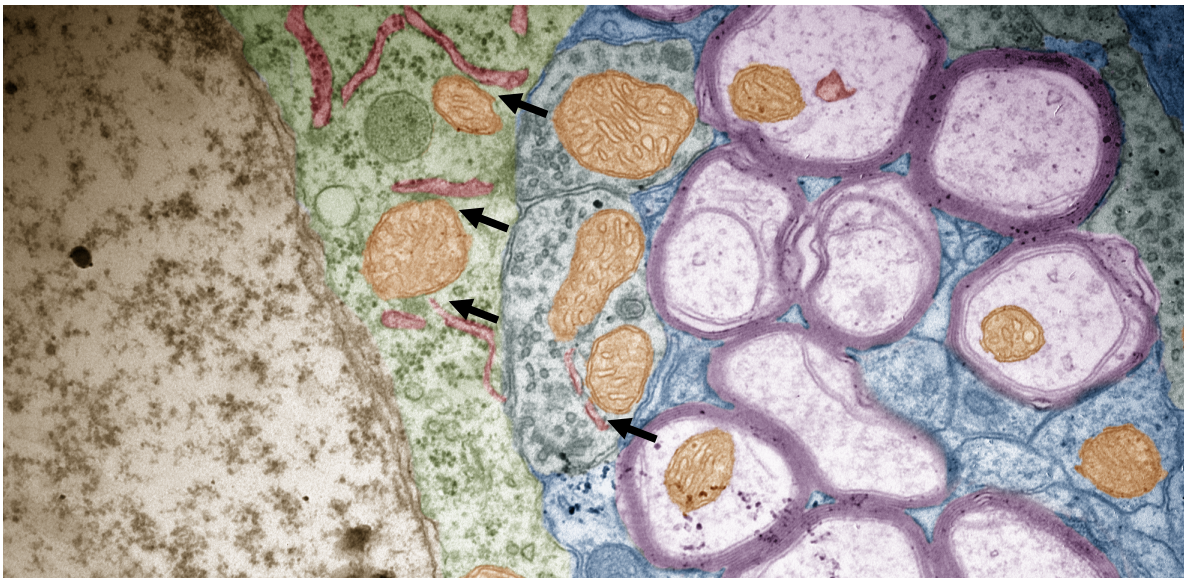

Figure 4. Electron micrograph of mouse pyramidal cell (CA1) next to several myelinated neuronal projections. Neuron is represented in yellow, mitochondria in orange, cytoplasm in green, endoplasmic reticulum in red, pre-synaptic terminals in grey and myelin structures in violet. Four close contacts between mitochondria ER can be seen where ER is in close proximity with mitochondria (black arrows).

1.3.2 Ultrastructure and tethering proteins of MERCS

Several years after discovering MERCS, the scientific community is still struggling to identify the proteins involved in the tethering of both organelles in superior eukaryotic cells. Several publications have tried to identify MERCS-related proteins with different techniques, including proteomics. In 2013, Poston performed, for the first-time, proteomics in a subcellular fraction enriched in ER-mitochondria contacts from mouse brain. They identified 1212 proteins and observed that most of the proteins in this fraction were suggested to be proteins from mitochondria and ER, and to be involved in mitochondrial functions and OXPHOS.[160] Two years later, Liu performed a similar study in rabbit skeletal muscle, identifying 459 proteins where almost 25% of these were also involved in cell metabolism.[161] Recently, Wang and colleagues have increased these numbers, since they identified over 2800 MERCS-proteins in both mouse and human testis, and around 2500 MERCS-proteins in mouse brain. In addition, they showed that these two tissues overlapped in 1993 MERCS-related proteins.[162] Two other studies have not only performed proteomics on untreated samples but they also compared MERCS-protein content during viral infection[163], in diabetes[164] and in caveolin-1 (pivotal regulator of cholesterol and component of MERCS) KO mice.[165] The mentioned proteomic studies are very promising since they have identified hundreds of possible candidates that could be

involved in the tethering between ER and mitochondria. However, most of these candidate proteins still need to be validated, which can be difficult due to the complexity of finding proteins that are exclusively located in MERCS.[166] However, by 2013, 75 proteins had been functionally connected to MERCS.[167] Nowadays, after 6 years, some new proteins have been identified and characterised but there are still several awaiting validation. Of these already identified and characterised proteins, some have been described to act as scaffolds, tethering the two organelles, while others perform biological functions and some can do both. In the next subsection, I will describe some of the most relevant MERCS-proteins for the MERCS ultrastructure.

1.3.2.1 Saccharomyces cerevisiae *(Yeast)*

In 2009, Kornmann and colleagues identified for the first time the ER-mitochondria encounter structure (ERMES), using a genetic screen to isolate yeast mutants.[168] ERMES is constituted of two OMM proteins (Mdm10 and Mdm34), one ER protein (Mmm1) and one cytosolic protein (Mdm12). All these proteins co-localize with MERCS and elimination of the ERMES complex lead to cell death. Moreover, elimination of any of the ERMES proteins decreased MERCS function connected to the conversion of PSer to PE and PC, suggesting that the ERMES formed a bridge between ER and mitochondria.[168] However, in 2012 a similar study showed that elimination of ERMES did not affect this conversion of PSer to PE.[169] Also, while the coupling between ER and mitochondria is dynamic and transient, the ERMES complexes are long-lived.[156,166,168] Therefore, it was proposed that ERMES has a role in mitochondrial morphology rather than a role in tethering ER and mitochondria.[166] The existence of orthologues of ERMES in mammalian cells is still unknown.

1.3.2.2 Mfn1 and Mfn2

In 2008, De Brito and colleagues showed, for the first time, the involvement of mitofusins in MERCS. They showed that the ER morphology was altered in mouse embryonic fibroblasts (MEF) cells lacking Mfn2 ($Mfn2^{-/-}$) and that Mfn2 was present in subcellular fractions enriched in MAM-OMM. Using volume-rendered 3D reconstructions of *z*-axis stack of confocal images and *in vitro* interaction assays, they concluded that MEF $Mfn2^{-/-}$ cells showed less interaction between ER and mitochondria. They also showed that while Mfn1 was localised only in the OMM, Mfn2 localised to both the OMM and MAM. In addition, they reported that these

proteins can form hetero- and homotypic complexes (Mfn1-Mfn2 and Mfn2-Mfn2, respectively), linking the two organelles **(Figure 6)**.[170] However, in 2012, Cosson and colleagues used TEM to evaluate if MERCS were altered in the same cells. Surprisingly, they reported an increase in the number of MERCS although the average length of MERCS was not changed.[171] In 2015, Filadi and colleagues reported similar results. To confirm whether the decrease of MERCS in MEF Mfn2$^{-/-}$ was due to clonal modification, they compared both knock-out (KO) and transient knock-down (KD) of Mfn2 in MEF using TEM. Both conditions showed an increase of close contacts (cleft distance ≤ 15 nm) between ER and mitochondria when compared to controls. Furthermore, they addressed the discrepancy between the confocal microscopy data from De Brito et al. and the TEM data from Cosson et al. and Filadi et al.[171–173] According to the authors, co-localization in fluorescence microscopy is affected by changes in organelle morphology. If the area of the organelles increased substantially more than the perimeter, this could result in an apparent decrease of ER-mitochondria tethering.[173] In fact, upon Mfn2 ablation, mitochondrial fragmentation and swelling is observed, making the classical co-localization methods (Mander's and Pearsson' coefficients) unsuitable. They further proved this by showing that there was an increase in Ca^{2+} shuttling from ER to mitochondria in Mfn2 KD cells, which was not observed in the original publication due to the decreased levels of mitochondria calcium uniporter (MCU).[171,173,174] In **Study III** we further confirm Cosson et al. and Filadi et al. data since we similarly show that KD of Mfn2 in HEK293 cells overexpressing APP^{Swe} also leads to an increase of MERCS length.[174] Other reports have assessed this issue but still, as of today, the scientific community has not fully agreed on the exact role of Mfn2 in MERCS due to the inconsistency of the results obtained by different groups. Even though down-regulation of Mfn2 has been one of the most used methods to modulate MERCS over the last few years, the conclusions deduced from the available data should have considered both possible roles of Mfn2.

1.3.2.3 VAPB and PTPIP51

Vesicle-associated membrane protein-associated protein B (VAPB) and protein tyrosine phosphatase-interacting protein 51 (PTPIP51) were first described to interact with each other and to modify intracellular Ca^{2+} concentration in 2012.[175] Currently, they are one of the most well described and characterised tethering pair of MERCS **(Figure 6)**. From the first publication, De Vos and colleagues showed

that VAPB was present in MAM and that different levels of PTPIP51 altered the co-localization of VAPB with mitochondria. Furthermore, depletion of either one of the proteins leads to changes in Ca^{2+} levels.[175] Two years later the same group showed that both WT and mutated Tar DNA-binding protein 43 (TDP-43) [a protein connected to amyotrophic lateral sclerosis/frontal temporal dementia (ALS/FTD)] disrupted MERCS and the interaction between VAPB and PTPIP51, via activation of glycogen synthase kinase-3β (GSK-3β).[176]

1.3.2.4 Other MERCS modifying proteins

Using bioinformatics and prediction software, Hirabayashi and colleagues identified PDZD8 (a functional orthologue of the ERMES protein Mmm1) as a possible MAM protein in metazoans. They identified that PDZD8 is located in MAM and not in the pure mitochondrial fraction. They also showed that PDZD8 is required for formation of MERCS since its ablation led to less interaction between ER and mitochondria and to less Ca^{2+} transfer from ER to mitochondria, leading to unbalanced Ca^{2+} dynamics in cortical neurons. The mitochondrial protein which interacts with PDZD8 remains to be discovered **(Figure 6)**.[177]

Transglutaminase Type 2 (TG2) is the most ubiquitous member of a large enzyme group that catalyses Ca^{2+}-dependent post-translational modification of proteins, including protein-protein cross-linking, incorporation of primary amines into proteins and glutamine deamination. Recently it was reported that TG2 interacts with Glucose-regulated protein 75 (Grp75) and in its absence the interaction between Grp75 and inositol 1,4,5-tris-phosphate receptor (IP3R) 3 (IP3R3) was increased. Using proximity ligation assay (PLA), immunocytochemistry and TEM the authors showed that in the absence of TG2 there were less interactions between IP3R3 and VDAC1, less overlap between ER and mitochondria and a decrease of MERCS.[178]

Curiously, unlike other MAM proteins, ATPase family AAA domain-containing protein 3 (ATAD3) has been described as an intermembrane tethering protein. ATAD3 spans from IMM to OMM and interacts with MAM.[179]

Phosphofurin acidic cluster sorting protein 2 (PACS-2) is a cytosolic protein that can modulate MERCS and depletion of this protein leads to decreased MERCS and to the block of apoptosis upon its induction.[180,181] Still today, it is unknown if this protein is a modulator or a tethering protein in this subcellular region. Mammalian target of rapamycin complex 2 (mTORC2) has been reported to be present in MERCS and it can activate PACS-2 via Akt pathway.[182]

1.3.3 Biological processes in MERCS

Several biological processes have been connected to MERCS. Here, I will describe some of the most studied and relevant ones for this thesis: mitochondrial dynamics, Ca^{2+} shuttling, apoptosis, phospholipid metabolism and autophagosome formation.

1.3.3.1 Mitochondrial dynamics

Interestingly, ER and MERCS have important roles in regulating mitochondrial dynamics. Recently, it was reported that ER defines the exact site where mitochondrial division occurs.[183] Firstly, there is the formation of MERCS, as ER surrounds the mitochondrial fission site. This allows the ER protein inverted formin-2 (INF2) and the mitochondrial protein actin-nucleating (Spire 1c and Arp2/3 complexes) to recruit actin-myosin assemblies. Next, Drp1 is recruited to the ER-marked places by the recruiting proteins and mitochondria undergo fission.[184,185] Interestingly, this event is thought to be controlled by mitochondrial matrix molecules since the constriction of the IMM occurs before the constriction of the OMM. In fact, actively replicating mtDNA is present in these ER-associated mitochondrial constriction and division sites, suggesting it could have a role during mitochondria fission.[186,187]

1.3.3.2 Ca^{2+} shuttling

One of the major functions of MERCS it to regulate Ca^{2+} shuttling between ER and mitochondria. Ca^{2+} is one of the most ubiquitous secondary messengers in the cell. Even small changes in intracellular Ca^{2+} concentrations mediate rapid response events, leading to drastic changes in cell functions (eg. muscle contraction, neuronal impulse and protein folding).[77,188,189] To allow these rapid and intense responses, Ca^{2+} levels in the cytosol are kept low by exporting this molecule to the extracellular milieu and by storing into different organelles (like ER). In response to specific stimuli, the existence of Ca^{2+} “hotspots” in the cell allows a precise spatiotemporal response to changes in Ca^{2+} levels. Upon stimuli, Ca^{2+} can be released from the different stores into the cytosol or by the opening of channels in the plasma membrane. Therefore, both Ca^{2+} concentrations and diffusion in the cell have to be highly regulated.

ER has been shown to be the major Ca^{2+} storage within the cell.[190] The majority of Ca^{2+} enters the ER from the cytosol via SERCA and is released by either

the ryanodine receptors or IP3Rs. Mitochondria, in turn, can also transiently buffer Ca^{2+} at a smaller scale by taking up Ca^{2+} from the cytosol and ER.[190]

The process of Ca^{2+} shuttling from ER to mitochondria has been well described. Firstly, Ca^{2+} is discharged from the ER via IP3R3 and shuttled to the IMS via VDAC1 supported by Grp75 **(Figure 6)**.[156,191] Three different types of IP3Rs receptors have been described: IP3R1, IP3R2 and IP3R3. IP3R3 has been shown to be highly enriched in MAM and therefore I mostly refer to the isoform 3 throughout this thesis.[192] Similarly, VDAC has different isoforms being VDAC1 the most abundant.[193] VDAC1 is a porin permeable not only to Ca^{2+} but also to energy-related metabolites like succinate, malate, pyruvate, NADH, ATP, ADP and phosphate, which are essential to keep mitochondrial homeostasis.[194,195] From the IMS, Ca^{2+} crosses the IMM into the mitochondrial matrix via MCU.[196] Due to the extreme impermeability of the IMM this is the rate limiting step of the entire shuttling process. Moreover, this also means that this shuttling requires an active OXPHOS and electrochemical gradient.[197,198] Interestingly, MCU has a very low affinity for Ca^{2+} which prevents the uptake of Ca^{2+} into mitochondria when the levels in the cytosol are low.[199,200] Therefore, the formation of Ca^{2+} hotspots is essential to guarantee that Ca^{2+} concentration is higher around MCU so it overcomes its low affinity.[201] Since prolonged overflow of Ca^{2+} into the mitochondrial matrix could lead to initiation of apoptosis, Ca^{2+} cations are removed from mitochondria by mitochondria sodium/Ca^{2+} exchanger (NCLX) and taken up into ER again via SERCA.[202] Since IP3R3, Grp75 and VDAC1 proteins are enriched in MERCS, it is believed that this is the major Ca^{2+} shuttling system for transfer of the cation from ER to mitochondria. Several proteins have been shown to modulate IP3R3-Grp75-VDAC1 protein complex, eg. Sigma-1 receptor is an ER resident chaperone that stabilises IP3R3 in MAM, prolonging the Ca^{2+} signalling between ER and mitochondria.[203,204] Another example is TOM70. In **Study IV** of this thesis we show that knock-down of TOM70 leads to reduction of Ca^{2+} shuttled from ER to mitochondria due to changes in the location of IP3R3 at MAM. This knock-down led to changes in mitochondrial respiration, cell number and autophagosome formation.[205] Using different artificial linkers with different sizes and tags for ER and mitochondria it was shown that the closer the organelles are the more efficient the uptake of Ca^{2+} by mitochondria is and vice-versa, strengthening the hypothesis that Ca^{2+} shuttling occurs here.[201]

Although Ca^{2+} is essential for mitochondrial functions, this organelle is highly sensitive to Ca^{2+} variations in the cation concentration due to several Ca^{2+} sensitive

proteins in OMM, IMM and mitochondrial matrix.[77,189] In fact, the constant shuttling of small amounts of Ca^{2+} from ER to mitochondria is essential to maintain mitochondrial function and ATP production. When the levels of Ca^{2+} are moderately increased in the mitochondria matrix, this leads to the activation of several dehydrogenases (eg. pyruvate dehydrogenase (PDH), alfa-ketoglutarate dehydrogenase, isocitrate dehydrogenase) in the TCA cycle, boosting ATP synthesis **(Figure 6)**.[155,166,206,207] One way that Ca^{2+} regulates PDH activity is through controlling its phosphorylation (inactive PDH) by PDH kinase and dephosphorylation (active PDH) by Ca^{2+} activated phosphatase.[77] However, prolonged overflow of Ca^{2+} into mitochondria leads to activation of programmed cell death mechanisms (expanded in the subheading **1.3.3.3 Apoptosis**) **(Figure 6)**. On the other hand, decrease of Ca^{2+} shuttling leads to a decrease in oxidative phosphorylation and cellular energy crisis.[208,209]

Therefore, regulation of Ca^{2+} levels and mitochondrial functions are closely connected. While, Ca^{2+} regulates mitochondrial function (and dynamics), mitochondria buffers local increase of Ca^{2+} levels and guides the molecule through the cell to coordinate cellular processes. This is extremely important for excitable cells like muscle fibres and neurons, which rely on Ca^{2+} to fulfil their main functions and require a strict spatiotemporal regulation.[77]

1.3.3.3 Autophagosome formation

Autophagosomes were first observed in the 1950s when several experts in electron microscopy found vesicles containing cytoplasmic organelles in different cell types but, it wasn't until 1963, that Christian de Duve introduced the term *autophagy*.[210] Nowadays, several types of autophagy have been described; some are specific (like mitophagy) and some are non-selective (like macroautophagy).[211] Macroautophagy, most commonly referred to as autophagy, has been widely studied and it is currently known that it is an intracellular degradation/recycling process that is highly conserved in eukaryotic cells and is essential for cell homeostasis and development. During specific stresses (like starvation), autophagy is activated and cytosolic material (such as proteins and/or organelles) is engulfed by a double-membrane structure, forming the autophagosome. This process allows the generation of free amino acids so they are available for protein translation in the stressed environment. The autophagosome is further matured and fused either with

multivesicular bodies or with the endosomal-lysosomal system, where the cargo can be degraded by lysosomal proteases.

Autophagosome formation can be divided in three phases: initiation, nucleation and expansion. Different proteins/pathways have been described to control autophagy initiation. One example is the mammalian target of rapamycin complex 1 (mTORC1). Under basal conditions mTORC1 is active, promoting cell growth and anabolic processes (eg. macromolecules synthesis), and inhibiting autophagy through several phosphorylation on unc-51-like kinase 1 (ULK1) complex. Another example is the AMP-activated protein kinase (AMPK). AMPK is activated in situations where levels of ATP decrease and the levels of ADP and AMP increase. Under starved conditions active AMPK stops anabolic processes and induces catabolic pathways, like autophagy, induced by inhibition of mTORC1 complex and phosphorylation and activation of ULK1 complex. ULK1 activity is then regulated according to the phosphorylation status of its different residues, either by mTORC1 or AMPK **(Figure 5)**.[211,212]

The activation of ULK1 complex will results in the formation of the isolation membrane via PI3KC3 complex that, together with several autophagy-related (ATG) proteins, leads to the maturation and formation of autophagosomes **(Figure 6)**. Microtubule-associated protein 1A/1B-light chain 3 (LC3B) is one of these proteins. LC3B is an ubiquitin-like protein that is synthesised in an unprocessed form (proLC3B). This form of LC3B is cleaved in its C-terminus into LC3B-I and then conjugated to PE forming LC3B-II. While other ATG proteins dissociate from the autophagosomal membrane before the closure of the isolation membrane, LC3B-II remains attached to both the inner and outer membrane of the autophagosome. Upon fusion with the lysosome, the LC3B-II in the inner membrane is degraded while the one in the outer membrane is recycled. Therefore, LC3B-II has been widely used to monitor autophagy since, unlike other proteins, it remains attached to the mature autophagosomes.[211–213] In order for LC3B to bind to the substrates that needs to be degraded (the cargo), proteins such as SQSTM1/p62 (p62) act as linkers between the ubiquitinated cargos and LC3B. Since p62 is captured together with the cargo and degraded in the autophagosome it can also be assessed as an index of autophagic activity, especially when autophagy is inhibited leading to p62 accumulation **(Figure 5)**.

Even though many of the proteins involved in autophagosome formation/maturation has been identified, it is still unknown exactly where the

membrane that originates autophagosomes (isolation membrane) arises from. Since the autophagosome formation is a fast process, it is believed that the cell has to be able to mobilise a substantial amount of membranes upon autophagosome inducing stresses. Although different studies have shown that the isolation of the membrane that originates autophagosomes can originate from Golgi[214,215], the plasma membrane [216] and even mitochondria[217], it is believed that the ER is the main source of the isolation membrane.[211] In 2003, Hamasaki and colleagues showed that upon starvation, ATG14L, DFCP1 and ATG5 were present in MAM-OMM enriched fractions, showing for the first time that autophagosome formation occurs at MERCS. They also showed that upon modulation of MERCS, by knocking-down of PACS2 and Mfn2, the number of autophagosomes was decreased.[218] This work was further confirmed by Garofalo and colleagues.[219] Recently, the MERCS tethering complex VAPB-PTPIP51 has also been shown to regulate autophagosome formation. Gomez-Suada and colleagues showed that decreased levels of these proteins led to the increased formation of autophagosomes and vice-versa. They also showed that this was connected to Ca^{2+} shuttling between ER and mitochondria.[220] Last year, another group showed that depletion of VAPA/B inhibits autophagy by impairing the maturation of the isolation membrane into autophagosome. They also showed that VAPA and B are recruited to the autophagosome formation sites during autophagy induction and that they interact with ULK1 and focal adhesion kinase family integrating protein (FIP2000), stabilising the ULK1/FIP200 complex.[221]

Interestingly, not only MAM seems to be essential for the formation of isolation membrane but also Ca^{2+} signalling has been shown to affect autophagy formation. Both mTORC1 and AMPK have been shown to be modulated by Ca^{2+} levels and thus different agents that alter Ca^{2+} signals have consequences in autophagosome formation and/or maturation.[222] Thapsigargin, for example, inhibits SERCA pump, preventing Ca^{2+} uptake from the cytosol to the ER leading to increased levels in the cytosol, triggering autophagy while chelating Ca^{2+} leads to autophagy inhibition.[222] Furthermore, also Ca^{2+} signalling in mitochondria has an important role in autophagosome formation. As mentioned before, shuttling of Ca^{2+} into the mitochondrial matrix is tightly regulated. Correct amounts of Ca^{2+} boost ATP production, while high concentrations lead to cell death. Besides, a decrease of Ca^{2+} shuttling from ER to mitochondria affects negatively affects the TCA cycle and ATP production and can lead to the activation of autophagy. AMPK activity is regulated by AMP:ATP ratio in the cell. During normal conditions the AMP:ATP ratio is low

(increased ATP levels), ATP binds to AMPK allowing phosphatases to dephosphorylate AMPK, thus making it inactive. On the other hand, increased levels of AMP:ATP lead to AMP binding to AMPK, protecting its phosphorylated form, making AMPK active.[223] Therefore, in conditions where Ca^{2+} shuttling to mitochondria is impaired, ATP levels drop, activating AMPK and inducing autophagosome formation, either through mTORC1 or ULK1/2 pathways.[222,224] Likewise, inhibition of IP3Rs using Xestospongin B, genetic knock-down, inhibition of IP3 production or mitochondrial Ca^{2+} uptake resulted, in basal conditions, in decreased mitochondrial Ca^{2+} and ATP production, triggering mTORC1-independent autophagy.[208,225] Curiously, treatment with Xestospongin B under starved conditions inhibited autophagy. Therefore, it is believed that IP3Rs-Ca^{2+} signalling can be pro-autophagy during normal conditions while in chronic starvation it acts anti-autophagy **(Figure 5)**.[226] Although the underlying mechanisms have not been unravelled, I personally think this is connected with MERCS. In order for autophagosomes to be formed, ATP is needed in different steps of the process, including initiation and progression.[227–229] Under basal conditions, where ATP is available, acute abolishment of Ca^{2+} shuttling to mitochondria induces cell stress and a decrease in ATP levels. However, conditions will still allow formation of autophagosome since there is still ATP available. During chronic starvation, due to the lack of nutrients, cells are already stressed, and the levels of ATP have probably dropped below normal. Therefore, the connection between ER and mitochondria is needed in order to shuttle Ca^{2+} to mitochondria so a boost of ATP in formed under this stress condition and allow autophagosome formation. Inhibition of this shuttling during starvation, through treatment with Xestospongin B, will then prevent the formation of ATP and, consequently, autophagosomes.

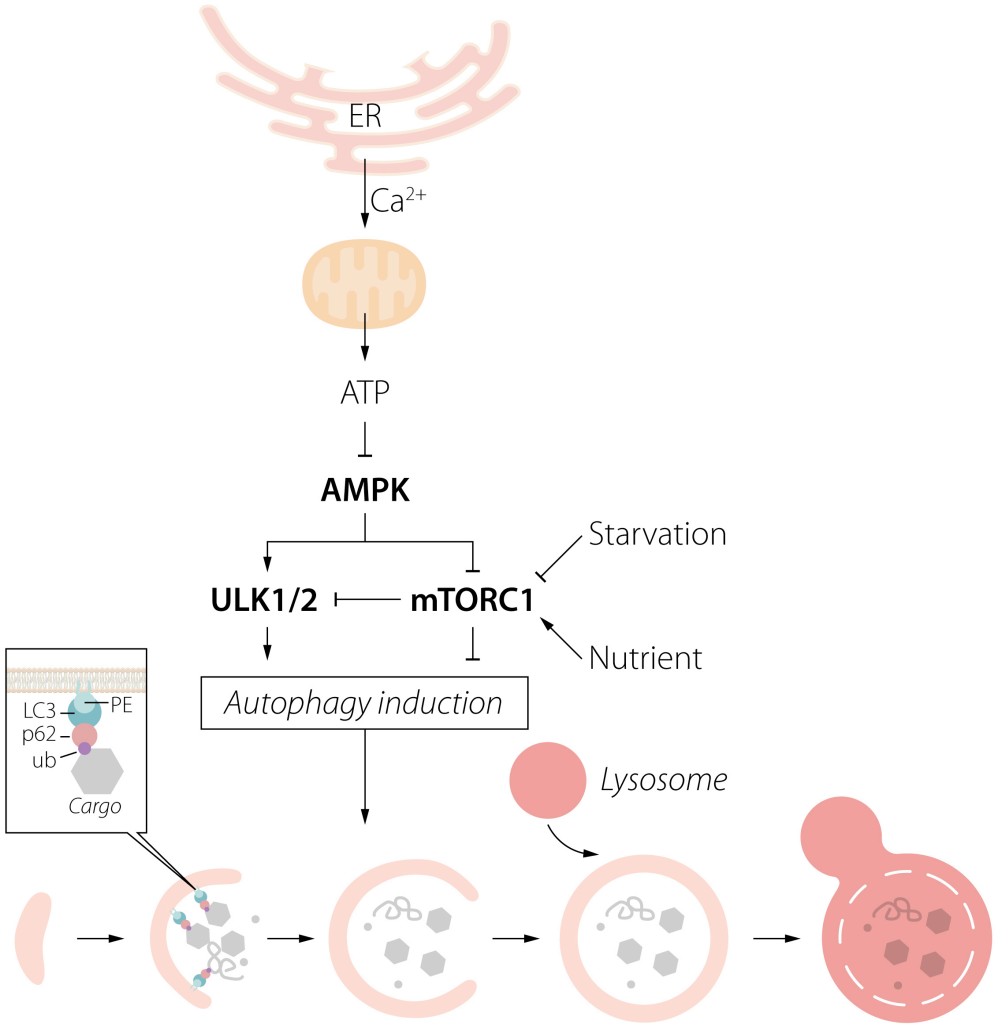

Figure 5. Schematic representation of MERCS affects autophagosome formation. Increased Ca^{2+} shuttle from ER to mitochondria leads to increase of ATP formation, which leads to inhibition of AMPK. Decrease in AMPK activity, on its turn, can either decrease ULK1/2 activity or increase mTORC1 activity. Ultimately, this results in induction of autophagy.

1.3.3.4 Apoptosis

As mentioned before, overload of Ca^{2+} into mitochondria can trigger apoptosis. Apoptosis, or programmed cell death, is a process where cells are eliminated in a structured manner. This mechanism is important for tissue development, to keep homeostasis by maintaining cell population, as a defence mechanism in immune responses and when cells are damaged.[230] Apoptosis can be induced by two different pathways: death receptor (extrinsic) or mitochondrial (intrinsic).[230,231] Most of the stimuli that trigger the mitochondrial apoptotic pathway induce changes in the IMM which, ultimately, leads to the opening of the mitochondria permeability transition (MPT) pore, loss of $\Delta\Psi m$ and release of pro-apoptotic factors to the cytosol (eg. cytochrome c, Smac/DIABLO, serine protease

HtrA2/Omi) which leads to activation of the caspase-dependent mitochondrial pathway. [230] Overload of Ca^{2+} has been shown to have several effects on mitochondria but ultimately it sensitises mitochondria, lowering the threshold for MPT pore opening.[231,232] Several lines of evidence have connected apoptosis to MERCS and increase of Ca^{2+} levels in mitochondria. Two independent publications showed that an increase of mitochondrial Ca^{2+} (through increase of MERCS) leads to an increase of apoptosis in RBL-2H3 cells and neurons.[154,233]

1.3.3.5 Phospholipid and lipids metabolism

The majority of organelles in the cell are surrounded by at least one phospholipidic monolayer. Since different lipids present distinctive features, alteration of these can lead to drastic changes in organelle function and integrity. Most of these phospholipids are generated in the ER and are distributed to the respective organelles by mechanisms that are still not completely known. Since phospholipids present long hydrophobic tails, they cannot be freely transported through the cytosol alone. Therefore, two options arise: they are either transported by vesicles or directly transferred to the target organelles through ER contact sites. As mentioned before, Vance and colleagues described in 1990 that certain phospholipids are formed in MERCS.[147,166] MAM is a lipid "raft"-like sub-region of the ER that shares several characteristics with bulk ER, including the presence of several proteins involved in lipid and phospholipid metabolism. Although, some of these common proteins are more enriched in bulk ER others are more enriched in MAM [eg. phosphatidylserine synthase-1 and -2 (PSS1/2)].[147,148,166] Although only a few proteins have been identified as a MAM specific protein as they are absent in bulk ER and mitochondria [(eg. phophatidylethanolamine-N-methyltransferase-2 (PEMT)].[234] PE is the major phospholipid constituent of the IMM and it has been reported to be formed by decarboxylation of phosphatidylserine (PSer) in mitochondria.[166] PSer is synthetize in the ER by MAM-enriched proteins PSer synthetase-1 and -2 (PSS1 and PSS2). PSer is then transported from the ER to the IMM where PSer decarboxylase (PSD) is located, forming PE which is shuttled back to ER. PE can then be converted to PC by PEMT and be shuttle back to mitochondria. Both PSS1 and PSS2 are highly enriched in MAM when compared to the bulk ER which suggests that PSer is synthesised and directly transported to mitochondria and not directly by cytosolic proteins or small vesicles.[166,235,236] Although, the exact mechanisms remain unknown.

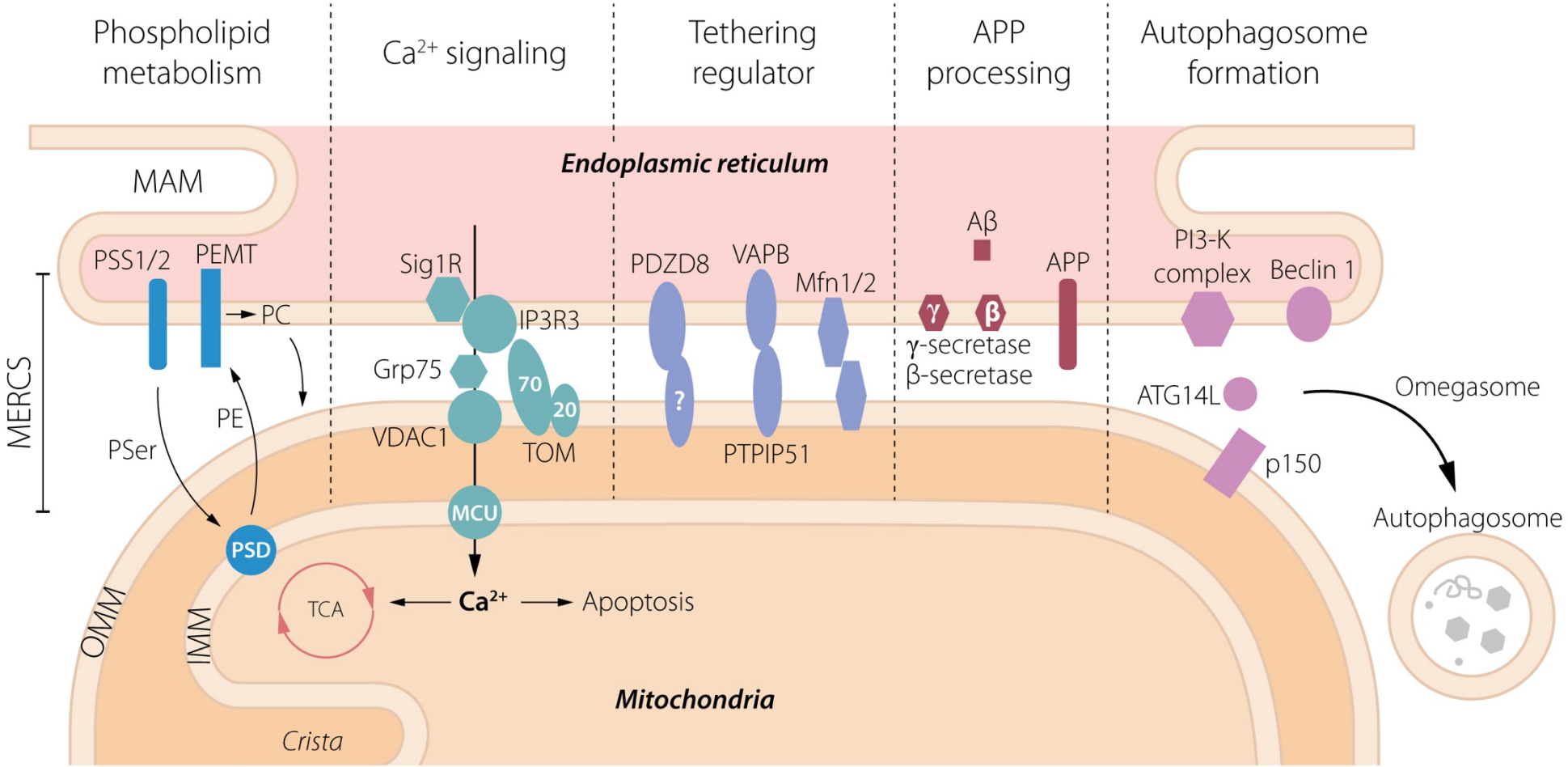

Figure 6. Schematic representation of MERCS ultrastructure and biological processes relevant for this thesis: phospholipid metabolism, Ca2+ signalling, APP processing, autophagosome formation. It is also represented some proteins involved in the tethering between MAM and OMM.

1.4 THE ROLE OF MERCS AND MAM IN DIFFERENT PATHOLOGIES

Due to all the biologic processes regulated at MERCS, alterations in these contacts structure and MAM function have been reported to be associated with different human diseases. In **Table 1** I have illustrated some examples:

Table 1 Examples of disorders associated with alterations of MERCS.

Disorder	Pathophysiological hallmarks	Pathology mechanism	MERCS role	Ref.
Parkinson's disease	Dopaminergic neurons from substantia nigra are lost. Tremors, bradykinesia, rigidity.	Mutation in PINK1 and Parkin leads to increased levels of Drp1 and mitochondria fragmentation and decrease of mitochondrial mobility.	Increase of mitochondrial Ca^{2+} and the average length of each contact, while the number of MERCS per mitochondrion were decreased.	237–240
ALS/FTD	Motor neuron dysfunction and frontal lobe impairment and dementia.	Deposits of TDP-43 fused in sarcoma (FUS) and dipeptide repeat proteins derived from C9ORF72 gene.	Reduced MERCS by disruption VAPB-PTPIP51.	176,241, 242
Cancer	Evasion of apoptosis, sustain proliferative signalising, active invasion and metastasis and changes in metabolism.	Increased levels of IP3R3 and VDAC1 were observed in glioblastomas and human papilloma virus-related cervical cancers. Decreased levels of Mfn2 were observed in gastric tumours.	Alterations in MERCS; Several oncogenes and tumour suppressors have been shown to localise at MAM and OMM.	192,243, 244
Obesity and type 2 diabetes	Alterations in insulin resistance and/or insulin secretion. Constant hyperglycaemia if not treated.	MERCS are altered in palmitate-induced insulin-resistance HuH7 cell and in liver of both obese and diabetic mice. Diabetic mice treated with anti-diabetic drugs showed improved insulin sensitivity and restored MERCS.		245,246

1.5 MERCS AND ALZHEIMER'S DISEASE

As for the disorders presented in the previous section, also dysregulation of MERCS has been shown in AD. In this subsection, I will summarise the studies which over the years, contributed to establishing a relationship between AD and MERCS.

1.5.1 β-secretase, γ-secretase, Aβ formation and MERCS

In the 2000's, Aβ was shown to be present in mitochondria in AD *post-mortem* brain.[247–249] However, whether Aβ was produced in mitochondria or translocated into mitochondria was still unknown. Between 2004 and 2011, our group and others described that APP, Aβ and γ-secretase are present in the OMM and that Aβ could be imported into mitochondria via the TOM complex and receptor for advanced glycation end products (RAGE).[247,250–253] However, it was still hard to explain how Aβ could be produced at the OMM since this membrane does not have the right biochemical environment to harbour these proteins (not a lipid raft) or to sustain β-secretase activity (due to the need to acidic pH). Moreover, it should be kept in mind that some of the data is derived from models with artificial expressions of APP and Aβ, and it is known that overexpression of proteins can lead to misallocation of proteins within the cell.[65]

It was not until 2009, when Area-Gomez and colleagues connected how APP processing and Aβ formation could occur so close to mitochondria. The authors identified, for the first time, that γ-secretase proteins, including PS1 and PS2, and APP were enriched in MAM-OMM subcellular fraction derived from mouse brain. PS1 was also found in plasma membrane/Golgi fraction, as reported before, but it was more enriched in the MAM-OMM fraction. Furthermore, they also showed that PS1 was 5- to 10-fold more enriched in MAM than in bulk ER.[54] Interestingly, γ-secretase was not only shown to be present here but also active, since AICD was found in MAM-OMM fraction but not in plasma membrane, pure mitochondrial and ER fraction. This data was further confirmed by co-localization between PS1 and fatty acid-CoA ligase 4 (FACL4, a MAM-enriched protein).[54]

Three years later, the same authors hypothesised that MAM was a detergent-resistant membrane and it has features of a lipid-raft, since APP, PS, Aβ and γ-secretase are enriched in lipid-rafts. Indeed, MAM was shown to be an intracellular detergent-resistant membrane and VDAC1 and PS1 integrity was not affected after treatment with Triton X-100. The authors also showed that the amount of cholesterol

in MAM was higher than in bulk ER and pure mitochondria. Furthermore, they observed that MEF cells lacking PS1 and/or PS2 showed an increase in total cholesterol, free cholesterol and cholesteryl esters when compared to WT. Interestingly, PS1 and PS2 double KO cells had a higher increase in these lipids, compared to single KO, showing that both PS can influence the formation of the cholesterol and other related molecules. To support these evidences, not only was phospholipid synthesis augmented in double KO cells but also in SAD and FAD patient-derived fibroblasts, including PSer indicating an increase in MERCS. Later, they observed that there is an increase in co-localization between mitochondria and ER, using fluorescence microscopy and, using TEM, an increase in long contacts (>200 nm length) and decrease in short contacts (<50 nm) in both PS dKO MEF and SAD/FAD-derived fibroblast. Lastly, they showed that Mfn2 KO MEF cells had a decrease in C99 and AICD levels.[53] **Study III** in this thesis confirms this data since we showed that acute knock-down of Mfn2 causes a decrease in Aβ levels.[174] However, while Area-Gomez and colleagues did not assess MERCS ultrastructure and assumed there was a reduction in MERCS, our assessment showed an increase of MERCS length. We know now, also from Gomez-Suaga and colleagues, that an increase of number and length of MERCS does necessarily represent an increase in MERCS-functions/activity.[174,220]

To further confirm the role of MERCS in Aβ metabolism we showed that a considerable amount of both Aβ40 and Aβ42 is formed in subcellular fractions enriched in MAM-OMM.[52] In comparison, mitochondria produced a very small amount of the peptide, strengthening the hypothesis that mitochondria do not produce a significant amount of Aβ. Two years later, Del Prete and colleagues uncovered more of the mechanism. They showed that β-secretase, γ-secretase, APP and Aβ are present and active in MAM-OMM-enriched fractions of SH-SY5Y cell lines, as well as in mouse brains. They further confirmed the location of these proteins and complexes in MERCS by showing that APP and its catabolites co-localized with mitochondria and ER using fluorescence microscopy. Finally, they showed an increase in MERCS in SH-SY5Y overexpressing APP with the Swedish mutation. [52,255] Even though there are evidences that Aβ is produced in MERCS, it is still not fully understood how the active γ-secretase is transported back to MERCS from the Golgi or endosomes. However, it has been shown that endosomes, under certain conditions, are in contact with mitochondria[256] and that the retrieval receptor

Rer1p has a role in transporting back the active γ-secretase from the Golgi to the ER, suggesting that active γ-secretase could actually be present in MAM.[257,258]

1.5.2 Mitochondria, ER and MERCS dysfunction in Alzheimer's disease

Even though it is widely accepted that the major hallmarks of AD are the amyloid plaques and NFTs, many other alterations have been connected with the disorder: altered Ca^{2+}[259], glucose[260], fatty acid[261], cholesterol [262] and phospholipid[263] homeostasis and metabolism, autophagosome formation and maturation[264,265] and mitochondria dysfunction.[266] Interestingly, all of these cellular functions have been connected to MERCS, as described previously. Additionally, MERCS have been described to be up-regulated in different non-neuronal AD models or in primary neuronal cultures incubated with a mix of Aβ species.[54,55,255] This, together with the role of MERCS in Aβ metabolism gave rise to the idea that MERCS alteration can be connected with AD.[54,167,267] Under the next subheadings, I will describe in more detail the affected biological processes relevant to this thesis: Ca^{2+} homeostasis, mitochondria dysfunction, autophagosome formation/maturation and, even though not relevant for this thesis, phospholipid and cholesterol metabolism since it was the trigger of the field and several publications have focused on these processes.

1.5.2.1 Ca^{2+} homeostasis

Although the exact mechanisms that lead to alterations in Ca^{2+} homeostasis in AD are unknown, however several studies have connected PS and Aβ as possible causes of this dysregulation. Since ER is the major Ca^{2+}-storage in the cells, it is believed that this organelle plays an important role under conditions of impaired Ca2+ homeostasis. Several evidences have emerged and tried to explain how PS can modulate Ca^{2+}-homeostasis in the cell. In the early 90's, Ito and colleagues showed that fibroblasts derived from asymptomatic members of AD families with PS mutation showed enhanced Ca^{2+}-release from the ER.[268] In fact, cells and tissues from animal models with mutated PSs were shown to activate IP3Rs, inducing Ca^{2+}-release.[269270,271] This was further confirmed when it was shown that reducing IP3R1 expression was able to rescue the exaggerated levels of Ca^{2+} in neurons and attenuated the Aβ and tau accumulation.[272] Another theory is that PSs can interact with SERCA, inducing overflow of Ca^{2+}-uptake into the ER[273] or inhibiting it.[274] More recently, Zampese and colleagues showed that, in SH-SY5Y cells and primary cortical neurons, the PS2-T122R mutation induces an increase in MERCS.[275] This increased connectivity between the two organelles led to an up-regulation of MERCS

functions, such as increased Ca^{2+}-shuttling from ER to mitochondria.[53,275] Interestingly, a new study from the same group showed that PS2 acts as a regulator of Mfn2, being able to "capture" Mfn2 and consequently regulate the contacts formed at MERCS.[157] Cells overexpressing WT PS2, but not PS1, showed an increase in MERCS, but only in the presence of Mfn2. Curiously, PS2 FAD mutation exacerbated these connections. However, Mfn2 antagonistic effect was independent of PS2. Moreover, PS2 and Mfn2 were shown to physically interact with each other while interaction with PS1 and Mfn1 was not observed. Therefore, I believe that PS2 can modulate Aβ formation not only through its pivotal role in γ-secretase but also by modulating MERCS.

Aβ, both extracellular and intracellular, has been shown to induce Ca^{2+}-dysregulation. For example, extracellular Aβ oligomers promotes influx of extracellular Ca^{2+} into the cell by forming Ca^{2+}-permeable pores and by activating Ca^{2+}-permeable channels.[276,277] Furthermore, incubation of primary hippocampal rat neurons with oligomeric Aβ leads to increase apoptosis, increase cytosolic and mitochondrial Ca^{2+} and, recently, it was reported that it enhances shuttling of Ca^{2+} from ER to mitochondria as well as co-localization between ER and mitochondria.[278,279] In addition, it was also reported that Aβ can induce Ca^{2+}-release from the ER, either by formation of pores in the ER membrane or enhancing Ca^{2+}-release via IP3Rs and RyRs, inducing Ca^{2+} leakage from ER.[280–282] Our group also showed that adding conditional media from CHO 7PA2 cells (enriched in different Aβ species) to WT primary cortical neurons upregulated the proximity between OMM protein VDAC1 and MAM protein IP3R3, as well as the shuttling of Ca^{2+} from ER to mitochondria.[55] Therefore, this data shows that both Aβ and PSs can affect Ca^{2+} homeostasis via MERCS.

1.5.2.2 Mitochondrial dysfunction

Mitochondrial dysfunction has also been described as one of the earliest events in AD due to the accumulation of Aβ in mitochondria prior to plaque formation. Even though the exact mechanisms of how Aβ affects mitochondria are unknown, several publications have suggested some of the following mechanisms: early in the pathology it has been shown that there is a reduced number of mitochondria[283]; reduced activities of TCA cycle enzymes, OXPHOS and ATP production[284]; a decrease in glucose metabolism[285] and an increase oxidative damage due to the augmented levels of ROS.[16,286,287] The latter alteration has been linked to both

impairment of complex I, which is connected with ageing and inhibition of complex IV by Aβ.[40,288,289] Increase of mitochondrial fragmentation has also been coupled to changes in the levels of mitochondrial dynamic proteins Mfn1, Mfn2, Opa1, Drp1 and Fis1.[266] Additionally, mitochondrial axonal transport and re-distribution in the perinuclear region of the organelle has been shown in AD patients cells and cell lines as well as alteration in mtDNA and mitochondrial transcripts.[40,266,290]

1.5.2.3 Autophagy

During ageing, there is a decline of the proteasome-mediated turnover of proteins. This implies that the cells start relying more on the autolysosome system to degrade proteins.[291] Recently it was shown that knock-down of Rubicon, a negative regulator of autophagy, increases lifespan and ameliorates age-associated phenotypes.[292] In fact, knock-out of autophagy-related genes in neurons leads to neuronal death marked by the accumulation of ubiquitin-positive aggregates, showing that autophagy is essential for neuronal homeostasis.[293,294] Most of the known autophagy mechanisms were identified in yeast and non-polarised mammalian cells and extrapolated to other types of cells. However, not much has been investigated in neurons nor neuronal cells. Since neurons are highly polarised cells it is believed that autophagy is regulated differently in these cells than in non-neuronal cells.[295–297]. For several years it was believed that autophagy was inactive in neurons unless it was induced. Today, we know that this is highly unlikely. As far as the production rate matches or is below the clearance rate by lysosomal degradation, autophaghosomes are rarely observed, giving the impression that autophagosomes are not formed in neurons. This has been confirmed by inhibiting basal autophagy clearance by cathepsins, which led to a rapid accumulation of autophagic vacuoles (AVs) showing that neurons keep the number of autophagosome low in basal conditions.[298,299]

The first evidences showing that autophagy was affected in AD were described by Nixon and colleagues when they observed autophagic vacuoles (AVs) in post-mortem AD brains, particularly within dystrophic neurites.[265] The accumulation of these immature AVs suggested that the retrograde transport and maturation of autophagosomes with lysosomes was impaired in AD and, therefore, the elimination of Aβ was impaired.[264,299] Curiously, these AVs were identified to contain full length APP, C99, PS1 and NCT, and to produce and accumulate intracellular Aβ.[300,301] Interestingly, PS1 has been shown to also regulate autophagy

by modulation of the shuttling of v-ATPase subunit to the lysosome from the ER.[302] However, the exact role of autophagy in Aβ metabolism remains unclear. While some reports claim that formation of Aβ can occur in the autophagosomes, other reports showed that autophagy impairment leads to a decrease in Aβ secretion, resulting in a decrease in amyloid plaques and to intracellular accumulation of Aβ.[57,301] In fact, pharmacological activation or inhibition of autophagy in SH-SY5Y leads to an increased in activity of α-, β- and γ-secretase and extracellular Aβ42 levels although γ-secretase activity and extracellular Aβ42 levels were more enhanced during the inhibition of autophagosomes. Curiously, no differences were observed in Aβ-cleaving enzymes.[303] In the same line of research, in **Study II** of this thesis, we show that models with high levels of Aβ show an increase in basal autophagy and that formation of autophagosomes occurs faster due to the increased in MERCS. Altogether, this data suggests a direct link between the amyloidogenic pathway and autophagy even though the exact mechanisms remain to be elucidated and different studies have shown contradicting results.

1.5.2.4 Phospholipid and cholesterol metabolism

As mentioned before, Area-Gomez and colleagues were pioneers in establishing a connection between MERCS and AD. While we and others have used mostly Ca^{2+} shuttling as a read-out for MERCS function, this group has focused on phospholipid and cholesterol metabolism. Recently, this group has shown that neuronal cells treated with astrocytes (APOE4) conditioned media showed an increased synthesis of PSer, PE and cholesteryl ester, and increase in co-localization between ER and mitochondria when compared to cells treated with conditional media from astrocytes (APOE3). Even though the authors do not identify the mechanisms behind ApoE4 modulation of MERCS they believe it is related with lipoprotein metabolism pathway. They also claim that this could be the mechanism where ApoE4 acts as a risk factor in AD.[304] The year after, the same authors showed that accumulation of C99 (by inhibition of γ-secretase) in MAM resulted in the upregulation of sphingomyelin hydrolysis by sphingomyelinases, resulting in increased in ceramide levels, impaired mitochondrial respiration and blocking of ceramide reversed the bioenergetics impairments.[305]

1.5.2.5 MERCS in Tau models

To the best of my knowledge, only two studies have connected tau protein to MERCS. In 2009, Perreault and colleagues showed that a mutant form of tau (JNLP3) increased the MERCS and, in 2018, Cieri and colleagues showed the same trend in the truncated caspase 3-cleaved 2N4RΔC20 tau protein, which induced fibrillation and seeding of WT tau.[306,307] Therefore, the role of tau in MERCs remains largely unknown. In **Study I** of this thesis we touch a little bit upon this topic, where we observe that while no correlation is found between tau ventricular levels and MERCS there is a decrease in the length of MERCS in patients with plaques and NFT.[308]

2 AIM OF THE THESIS

Altogether, the presented data strengthens the importance of mitochondria and MERCS in AD. Although several studies have established a connection between AD and MERCS ultrastructure and function, it is still unknown how MERCS alterations can lead to the pathology or how the pathology can lead to the alterations in MERCS, leading to the proverbial “the chicken or the egg” situation. The current state of the art in the field is:

- Both mitochondria and MERCS structure and function as well as autophagosome formation/maturation are altered in AD;
- APP, Aβ, β- and γ-secretase are present in subcellular fractions enriched in MAM-OMM; Aβ is also formed here;
- Autophagosomes form at ER-mitochondria contact sites and modulation of MERCS leads to changes in autophagosome biogenesis;
- Alteration in MERCS dynamics alters mitochondrial Ca^{2+} content and its function and dynamics;
- MERCS tethers in yeast are well described but in mammalian cells new scaffolds need to be further identified and explored, including regulator proteins;

Therefore, the aim of this thesis was to further elucidate the connection between MERCS, Aβ metabolism and autophagosome formation in AD. The dysregulation of mitochondria-ER contact sites may be a key step and a possible target in the cascade of events leading to neurodegeneration in AD.

The specific aims for each study were:

- **Study I**: study MERCS ultrastrastructure in human brain biopsies and establish a correlation between MERCS ultrastructure in patient diagnosed with dementia and other clinical parameters.
- **Study II:** study if Aβ affects MERCS ultrastructure and function in neuronal model, and how this led to changes in mitochondrial function and autophagosome formation.
- **Study III:** study if modulation of MERCS altered Aβ levels.
- **Study IV:** study if TOM70 has a role in MAM-OMM structure and function.

3 METHODOLOGY AND METHODOLOGICAL CONSIDERATIONS

3.1 MOUSE MODELS

Several AD mouse models have been developed over the past. Most of these models were developed using the mutations associated with FAD. Recently, a new "generation" of animal models have emerged. While the "old" mouse models relied on overexpression of humanised APP and/or PS (with or without mutations), the new models tried to obliterate the secondary effects of protein overexpression (like mislocalisation of proteins and unspecific binding). The new models developed by Prof Takaomi Saido and Dr Takashi Saito rely on knock-in (KI) of humanised APP instead of its overexpression. In this thesis, we analysed the number of MERCS and length as well as number and perimeter of mitochondria in one overexpression transgenic model ($APP^{Swe/Lon}$) and two KI models (APP^{NL-F} and APP^{NL-G-F}). The transgenic mouse model presented a humanised APP with both the Swedish and the London mutation (fixed tissue from the animals and respective controls were a kind gift of QPS Austria), while the KI model APP^{NL-F} presented both the Swedish and Iberian mutation, and the model APP^{NL-G-F} presented the same mutations plus the Artic mutation.

In general, these mutations lead to increase production of Aβ (specifically Aβ42 and Aβ40) except the Artic mutation (within the Aβ sequence), which increases the propensity and rate of Aβ40-aggregation. In **Study II**, in order to understand how the progression of the pathology could affect MERCS we analysed hippocampus (CA1) and cortex from animals of four (WT and $APP^{Swe/Lon}$) and three (APP^{NL-F} and APP^{NL-G-F}) different ages (2, 4, 6.5 and 10 months and 4, 6.5 and 10 months respectively), respectively (total of 48 animals). In brief, anesthetised animals were directly perfused with 2% glutaraldehyde and 1% formaldehyde in 0.1M phosphate buffer and brains dissected and hemisected. Right hemisphere was put in fixation solution overnight and the next day placed in brain slicer matrix and CA1 and cortex collected and processed for ultrathin sectioning for ultrastructural TEM. The rest of the brain was kept in fixative solution at 4°C.

3.2 HUMAN BRAIN BIOPSIES

Idiopathic normal pressure hydrocephalus (iNPH) is a neurological disorder characterised by cognitive impairment as well as enlarged cerebral ventricles. Even though the mechanisms behind this pathology are unknown, this disorder shares comorbidity with AD, including the presence of the major AD hallmarks: amyloid plaques and NFTs.[17,309] For further information about iNPH please refer to Williams et al.[309] The only treatment available for iNPH is the introduction of a CSF shunt which alleviates the pressure and the symptoms.[310] For **Study I**, cortical biopsies were obtained during this procedure as previously described.[311] Briefly, the sample was collected from cortex, localised three centimetres laterally from the midline close to the coronal suture, and placed in fixative solution (1% glutaraldehyde and 3.7% formaldehyde in sodium phosphate buffer) up to 10 minutes after collection for TEM. Samples were then sectioned and stained with haematoxylin-eosin and immuno-histochemistry performed with phosphorylated tau antibody (MN-1020, clone AT8 IGH 135, Thermofisher) and Aβ (6F/3D, M0872; Dako) to check for and to characterize amyloid plaques and NFTs. Samples were then stratified in groups according to dementia diagnosis or absence/presence of amyloid plaques and NFTs.

Table 2 Clinical data from iNPH patients

	#	Gender	Age	Comorbidities	MMSE
$A\beta^-/tau^-$	1	F	75	NI	22
	2	F	76	NI	23
	3	F	77	NI	25
	4	M	75	NI	19
$A\beta^+/tau^-$	5	M	86	LBD/VaD	13
	6	F	79	NI	24
	7	M	79	NI	19
	8	F	71	NI	20
	9	F	76	NI	23
$A\beta^+/tau^+$	10	F	78	NI	23
	11	F	77	NI	28
	12	F	74	NI	24
	13	M	79	AD/VaD	14
	14	F	81	AD	15

3.3 HUMAN *POST-MORTEM* FRONTAL CORTICES

Post-mortem frontal cortices were obtained from the Karolinska Institutet Brain bank for **Study II**. A total of 4 non-demented controls and 4 patients carrying the FAD APP^{Swe} mutation were used. Frozen tissues were cut in small pieces and homogenised in a $5cm^3$ glass-Teflon (1200rpm, 12 strokes) in digestion solution (RIPA, PIC and PPI). After homogenisation samples were left on ice for 10 min and quickly homogenised again and spun down at 7000g for 10 min at 4^oC degrees to remove debris. Supernatant was collected and protein concentration determined by BCA. All the procedure was done on ice.

Table 3 Clinical data from *post-mortem* FAD patients

	#	Age of death	Sex	PMT	Clinical diagnosis	Age of onset
Control	1	82	F	9h	CV	-
	2	80	M	16h	CV	-
	3	67	M	21h	CV	-
	4	68	M	27h	CV, pneumonia	-
APP^{Swe}	1	62	M	40h	AD	53
	2	66	M	24h	AD	61
	3	56	M	24h	AD	44
	4	62	F	24h	AD	51

PMT – post-mortem time, **CV** - cardiovascular

3.4 CELL MODELS

Two different immortal cell lines were used in this thesis: Human embryonic kidney (HEK) 293 cells [(WT and stably expressing human APP^{Swe} (gift of Dr Johan Lundkvist)] for **Study III** and mouse embryonic fibroblasts (MEF) cells for **Study IV**. Both cell lines were grown in DMEM supplemented with 10% Foetal Bovine Serum (FBS). Cells were grown in T75 flaks and split every 2-3 day when confluency was between 70 to 90%. No antibiotics were used except for HEK293 APP^{Swe} where Zeocin (100µg/mL) was used to preserve the selection.

For **Study II** primary neuronal cells (PCN) derived from E15-16 mouse embryonic cortices were used. PCN were seeded in Poly-D-lysine coated plates in Neurobasal supplemented with 1% (%V/V) L-Glutamine and 2% B-27, media

changed after one week (half of conditional media and half of new media) and usually treated/lysed/fixed at DIV13-15 were.

3.5 CELLULAR EXPOSURE TO AMYLOID β-PEPTIDE

Aβ1-42 (AnaSpec, #AS-24224) was prepared according to the manufacturer's instructions to a stock solution of 5mM. Monomeric and oligomeric Aβ42 were prepared according to [312] and added to PCN for 24 hours (2μM) with or without scFvA13 (40nM). The intrabody scFvA13 was used to target oAβ and silence its function.[313] Monomeric Aβ42 was prepared by directly dissolving stock Aβ42 to final concentration of 1mM. Oligomeric Aβ42 was prepared in the same way but left at 4°C for 24h and then added to the cells.

3.6 CELL STARVATION AND AUTOPHAGOSOME QUANTIFICATION

Amino acid starvation has been widely used as a method to induce autophagy. PCN were deprived from amino acids and serum by changing the growing media (Neurobasal supplemented with L-glutamine and B-27) to Early's Balance Salt Solution (EBSS) for 30 minutes, 1h, 1h30, 2h, 2h30 or 3h.

Upon starvation, autophagosome were quantified by measuring LC3-I and LC3-II levels. The conjugation system LC3 has been widely used as an indirect measure of the amount of autophagosomes since it is the only protein that binds to the phagofore and remains attached during the matured autophagosome, only being degraded when autophagolysoosme is formed. LC3 exists in two forms: LC3-I and LC3-II (the last one is the lipidated form conjugated with PE). Only the last form integrates into autophagosome membrane, thus the levels of this protein are an indirect measure of the amount of autophagosomes. LC3-II form is larger in mass than LC3-I but it migrates faster in SDS-PAGE gels due to its increase dhydrophobicity.[213]

3.7 TRANSMISSION ELECTRON MICROSCOPE, MITOCHONDRIA PROFILE AND QUANTIFICATION OF NUMBER AND LENGTH OF MERCS

Due to the proximity between MAM and OMM (<80 nm) conventional fluorescent microscope, which has a resolution limit of 200 nm, cannot be used. Therefore, Tecnai 12 BioTWIN TEM (FEI company, Eidnhoven, The Netherlands) was used in all four studies of this thesis to quantify number and length of MERCS, number and perimeter of mitochondria profiles and number of MERCS per mitochondrion. Samples started by being chemically fixed: brain biopsies in 1%

glutaraldehyde and 3.7% formaldehyde, mouse brain in 2% glutaraldehyde and 1% formaldehyde and cell lines and primary cortical neurons in 2.5% glutaraldehyde [% calculated using (V/V)]. The different ratios used were due to different features of the fixatives. While glutaraldehyde has a strong fixative power its diffusion throughout the sample is slow, therefore it is used for flat or thin samples (eg. cell cultures). For tissue samples, since it is required that the fixative diffuse faster to keep the integrity of the tissue for imaging, a mix of glutaraldehyde and formaldehyde is used. Even though formaldehyde has a weaker fixative power it diffuses quicker through the tissue, keeping its integrity while the glutaraldehyde slowly diffuses to the core of the tissue. After fixation, cells are incubated for 2h with 2% OsO_4 and dehydrated in ethanol and acetone. They are then embedded in LX-112 resin (Ladd, Burlington,VT, USA) and ultrathin sections made using Leica Ultracut UCT (Leica, Wien, Austria). Samples are then contrasted with uranyl acetate followed by lead citrate and observed in TEM (100kV) and digital pictures taken with Veleta camera (Olympus Soft Imaging Solutions, GmbH, Münster, Germany) at a magnification of 20.500X. Number and length/perimeter of MERCS and mitochondria profiles was then calculated using the freehand tool at ImageJ software (National Institutes of Health, Bethesda, MD, USA). A MERCS was considered if the distance between ER and mitochondria is equal or small than 30 nm.

3.8 ALAMARBLUE

30 minutes before finishing cell treatment (siRNA or starvation), alamarBlue™ Cell Viability Reagent (resasurin, DAL1025, ThermoFisher) was added into the cell media and cells put back at 37°C, 5% CO_2. Media, together with the alamarBlue™ reagent, were transferred to a black 96-well plate with a clear bottom and fluorescence measured (λ_{Ex}= 530-560nm; λ_{Em}= 580-610nm). Cells were lysed, protein determined and fluorescent arbitrary units normalised to protein levels.

3.9 MITOCHONDRIAL TOXGLO

Cell membrane permeability to necrotic proteins and ATP levels were measured using the Mitochondrial ToxGlo kit (Promega) according to manufacturer's protocol. MEF and HEK293 APP^{Swe} were seeded in 96-well black plates with a clear bottom and siRNA treatment performed. After 48h, media was removed and the cytotoxicity reagent was added for 30 min at 37°C and fluorescence measured (λ_{Ex}= 485nm; λ_{Em}= 525nm). For ATP levels the ATP detection reagent was added and luminescence measured.

3.10 SEAHORSE

WT derived PCN were grown in Seahorse XF96 microplate (#101085-004, Agilent) (26.000 cells per well) for 14 DIV. On 15 DIV, cells were starved with EBSS as before. During the last 30 minutes, cells were calibrated in media (pH=7.2-7.4) without sodium bicarbonate and phenol red, at 37°C and without CO_2. Basal respiration and ATP production was then measured using Seahorse XFe96 Analyser (Agilent), according to Seahorse XF Cell Mito Stress Test Kit (Agilent) (final concentration: 1 μM Oligomycin, 1 μM FCCP, 0.5 μM Rotenone and Antimycin A) and values obtained normalised to the amount of protein.

3.11 AEQUORIN CALCIUM MEASUREMENTS

Apoaequorin, in the presence of the co-factor coelenterazine, originates aequorin. In the presence of Ca^{2+} this molecule induces the transformation of coelenterazine to coelenteramide, inducing coelenteramide release as well as the emission of photons. These photons can be detected by a photomultiplier (H7360-01 Hamamatsu Photonics), the output of the amplifier/discriminators was then captured by a photon counter (C8855-01 Hamamatsu Photonics) and recorded for analysis by the software (Hamamatsu Photonics), allowing to determine the concentration of Ca^{2+} that bound to aequorin. Ca^{2+} concentration were calibrated as described before.[314] In addition, by putting different protein tag in aequorin we can target the protein to different organelles in the cell.

HEK293 APP^{Swe} were seeded in poly-D coated coverslips and, after 24h, Mfn2 siRNA treatment performed like mentioned before. Furthermore, cells were also co-transfected with either cytosolic or mitochondrial aequorin cDNA. After 47h, coelenterazine (5μM) was added to the cells for 1h and coverslips put in the perfusion chamber and perfused with Krebs-Ringer modified buffer (KRB; 135mM NaCl, 5 mM KCl, 400μM KH_2PO_4, 1mM $MgSO_4$, 20mM HEPES, 11mM Glucose, pH = 7.4, 37°C) supplemented with 1mM of $CaCl_2$. Ca^{2+} release from ER was induced with a mix of ATP (100μM) and carbachol (CCH, 300μM) in KRB solution without $CaCl_2$ and in the presence of EGTA (600μM). Cells were lysed and the remaining aequorin pool discharged using digitonin (100μM) in a hypotonic Ca^{2+}-rich solution [$CaCl_2$ (10mM) in H_2O].

3.12 NEPRILYSIN ACTIVITY

HEK 293 APP^{Swe} cells were treated with siRNA as described before. After 48h, they were washed with 0.1 M MES (pH = 6.5) and incubated with substrate mix: protease inhibitor (#786-331; G-Biosciences, Maryland Heights, USA), 1 μM Z-Leu-Leu-Leu-H (aldehyde) (#3175; Peptide Institute, Osaka, Japan), 0.5 mM Suc-Ala-Ala-Phe-MCA (#S8758; Sigma-Aldrich, St. Louis, USA) with or without 10 μM thiorphan (#T6031; Sigma-Aldrich) for 1 hr at 37°C. Cells were then incubated with 15 μM Phosphoramidon (#4082; Peptide Institute) and 0.14μL of LAPase (#L5006; Sigma-Aldrich) and reaction was stopped with 10 nM of EDTA and fluorescence was measured in a plate reader (λ_{Ex} = 355 nm, λ_{Em} = 460 nm). Differences between substrate mix with and without thiorphan represent neprilysin activity.

3.13 SUBCELLULAR FRACTIONATION

Subcellular fraction was performed according to Hedskog et al. and Schreiner et al.[55,315] Briefly, brains of 2-4 months old female mice were collected, dissected into smaller pieces and homogenized (1500rpm, 12 strokes). Part of this homogenization was collected (whole homogenate - WH) and kept on ice. First centrifugation was performed at 800*g* (5min), pellet discarded and centrifuged again to remove nuclei and cell debris (pellet). Supernatant was collected and centrifuged at 9000*g* (10min) and crude mitochondrial was obtained in the pellet and supernatant collected. Supernatant was further centrifuged at 20.000*g* (20min), pellet (plasma membrane) discarded and supernatant centrifuged again 100.000*g* (1h) to generate the fraction containing ER. The crude mitochondrial fraction was put onto a 30% Percoll gradient and centrifuged at 95.000*g* for separation of pure mitochondria (bottom orange fraction) and MAM (top cloudy white fraction). Samples were then solubilized in RIPA buffer (30 min), unsolubilized material spun down (4000*g*, 5 min) and protein levels measured with BCA. Protein levels were assessed by WB. The whole protocol was done on ice or at 4°C.

3.14 PROXIMITY LIGATION ASSAY AND FLUORESCENCE MICROSCOPY

MEF cells were seeded in the inner ring of a Poly-D-lysine coated 35-mm glass-bottom plate (MatTek Corportation #P35G-0-20-C) for 24h. Cells were then fixed in 4% (V/V) formaldehyde solution and permeabilized with 0.4% CHAPSO. PLA protocol was then performed according to Duolink PLA kit (Sigma-Aldrich). Briefly, cells were blocked with Duolink blocking solution for 30 min at 37°C, antibody pairs (IP3R3-TOM70 or IP3R3-TOM20) incubated for 1h at 37°C. Positive and negative

controls were incubated with just one of the antibodies (IP3R3, TOM70 or TOM20). Specific plus and negative secondary antibodies bound to PLA oligonucleotides probe were added to the cells and incubated for 1h at 37°C. Positive controls were targeted with positive and negative probe of the same secondary species while negative controls were treated similarly to samples. The oligonucleotides on the PLA probes were then ligated (30min, 37°C), forming a DNA template that was amplified with fluorescence probes (100min, 37°C). F-actin was labelled with Alexa Fluor 488 Phalloidin (Thermo Fisher Scientific) and samples mounted using Duolink Mounting Media with DAPI. Fluorescent dots were then observed with a confocal microscope (Nikon A1RSi point scanning confocal inverted microscope). Images were acquired using a 60x oil immersion objective and PLA signals quantified with Duolink ImageTool.

3.15 STATISTICS AND CORRELATIONS

All data as analysed using IBM SPSS Statistics 22 software (IMB Corportation). Data was evaluated for normal distribution using Kolmogorov-Sminorv test, Q-Q plot and histogram distribution; to compare two groups, if normally distributed, samples were compared by independent-samples t-test for statistical significance and, if not normally distributed, samples were compared by non-parametric Mann-Whitney *U*-test. More than to two groups were compared with One-Way ANOVA. Unless stated all the values are expressed as mean ± SEM, n = corresponds to the number of independent experiments or individual samples/patients and each dot represents either the average of one independent experiment or an individual measure, * $p < 0.05$ were considered to be significant, ** $p < 0.001$.

3.16 ETHICAL CONSIDERATION AND ETHICAL PERMITS

All the studies in this thesis followed the declaration of Helsinki, the current European Law (Directive 2010/63/EU), local ethical board and the guidelines of Karolinska Institutet (KI) or of the university where the study/material was performed/obtained. For the animal work performed at KI in **Study II** and **Study IV** (WT, APP^{NL-F} and APP^{NL-G-F}) we used the ethical permit S53-14 and ID 407, $APP^{Swe/Lon}$ animals and respective controls were obtained from QPS Austria conformed the Austrian guidelines for the care and use of laboratory animals. For human post-mortem tissues (**Study II**) the study was done together with the KI Brain Bank under the ethical permit (2011/962-31/1) and for brain biopsies the Finnish ethical permit 5//2008 was used (**Study I**).

4 RESULTS AND DISCUSSION

Four studies originated from this doctoral project. In the next section, I will describe the major conclusions and contributions of these studies to the field.

4.1 STUDY I: ALTERATIONS IN MITOCHONDRIA-ENDOPLASMIC RETICULUM CONNECTIVITY IN HUMAN BRAIN BIOPSIES FROM IDIOPATHIC NORMAL PRESSURE HYDROCEPHALUS PATIENTS.

Mitochondria contact sites (MCS) and MERCS have previously been visualised in several tissues, however no one had, to the best of our knowledge, shown their ultrastructure in the human brain. In this study, we started by showing the presence of several mitochondria contact sites in human biopsies, including interactions with plasma membrane, nuclei, Golgi apparatus and lysosomes. The most common MCS observed were MERCS where 12.8 ± 0.5% of the mitochondrial surface was in contact with ER. We observed different types of MERCS; while some were just a punctate contact, others appeared as longer stretches. We also reported, for the first time, the presence of these MERCS in both pre- and post-synaptic terminals in human brains. Although the presence of MERCS in synapses has been described before in neurons from mice, the role of this subcellular region in synapses is still unknown.[47] Currently our laboratory is studying the possible role of MERCS in synaptic dynamics. We hypothesise that both mitochondria and ER, as well as their interplay, have pivotal roles in synapses due to their importance in regulating Ca^{2+} and ATP homeostasis.

Furthermore, in this study we showed that the number of MERCS was significantly increased in patients diagnosed with dementia [Lewy body dementia /vascular AD (VaD), AD/VaD or only AD], while the MERCS length was not altered. Similarly, the number and length of MERCS were also increased (even though not significant) in the group of patients with moderate or severe cognitive impairment (MMSE score was equal to or below 22) as compared to the no significant cognitive impairment group. In addition, the number of MERCS positively correlated with patient's age. Although some significant data was obtained with this sample size, we believe that an increased sample size could lead to a better understanding how MERCS ultrastructure changes with the decrease of cognitive functions and ageing. Nevertheless, we show that the juxtaposition between mitochondria and ER increases with ageing and with dementia progression however, it is hard to distinguish between the effect of ageing itself and of dementia. In order to assess

this, access to samples from patients with the same age but different cognitive diagnosis would be required.

Since it has been shown before that Aβ is formed in MERCS and that modulation of MERCS leads to changes in Aβ levels we believe that MERCS and Aβ formation are intrinsically connected. In this study, we observed a positive correlation between the number of MERCS and the ventricular levels of Aβ42, while no correlation was found with lumbar Aβ42 or with ventricular/lumbar total tau or phosphorylated tau (data now shown). Surprisingly, when we stratified the samples by the absence or presence of amyloid plaques and neurofibrillary tangles, we observed that MERCS length was decreased in the presence of both plaques and the tangles. Moreover, the % of distribution of MERCS was also shifted while patients without plaques and tangles had more than 50% of their MERCS length above 200nm and 22.7% of below 50nm, patients with both plaques and tangles had 26.9% of MERCS length above 200nm and 40.5% of contacts below 50nm. In conclusion, we show, for the first time, the presence of MCS in human brain biopsies and that MERCS ultrastructure is increased in demented and aged patients. This study contributes to a better understanding of MERCS dynamics in human brain, both in pathological and non-pathological situations.

4.2 STUDY II: AMYLOID β-PEPTIDE INCREASES MITOCHONDRIA-ER CONTACTS AND AFFECTS MITOCHONDRIAL FUNCTION AND AUTOPHAGOSOME FORMATION

Although several studies have connected alterations in MERCS with AD the underlying mechanisms were not elucidated. Additionally, most of these studies used cells derived from AD patients with complex cellular alterations and overexpressing models (which can have mislocalisation of APP protein and other secondary effects) or even non-neuronal cell models (which either do not have functional mitochondria – Warburg effect – or are not exclusively OXPHOS-dependent like neurons).[53–55,275,305] Therefore, in this study we aimed to determine the effect of Aβ on MERCS protein expression and/or ultrastructure in *post-mortem* FAD brain; cortex and CA1 of APP^{Swe}, APP^{NL-F} and APP^{NL-G-F} mouse models; and primary cortical mouse neurons treated with difference Aβ species.

We started by analysing the expression of MERCS proteins in *post-mortem* frontal cortex of FAD patients carrying the Swedish mutation. We selected this mutation because, as mentioned before, it leads to an increase of Aβ levels, more

specifically Aβ40 and Aβ42. We discovered that both Mfn1 and Mfn2 levels were significantly decreased as well as TOM70, cytochrome c and TIM23 levels. This data supports previous data showing an increase in mitochondria fragmentation in SAD[266] and suggests that MERCS ultrastructure/function could be altered. However, since we did not have access to fixed *post-mortem* samples, we decided to assess MERCS ultrastructure in other models. We decided to use three different mouse models that, similarly to the *post-mortem* samples, presented increased levels of Aβ: the overexpressing model APP^{Swe} and the KI models APP^{NL-F} and APP^{NL-G-F}. Overall, all these models showed an increase in the number of MERCS per mitochondria. Furthermore, the KI models also showed an increased number of mitochondrial profiles and da ecrease of the mitochondrial perimeter, suggesting that mitochondria are more fragmented in these models. Similar results were found in PCN derived from WT and APP^{NL-F}, where the KI model derived PCN showed an increased number and length of MERCS, as well as an increased number of mitochondrial profiles. In addition, preliminary data showed that also MERCS-related proteins (eg. Mfn2) are also altered also in APP^{NL-F}-derived PCN. We further confirmed this data by incubating WT PCN with synthetic Aβ42 (both monomeric and oligomeric, mAβ42 and oAβ42 respectively). Similarly, as observed in the APP^{NL-F} derived PCN, we observed an increase in the number of MERCS upon incubation with mAβ42. However, when normalising the number of MERCS to number of mitochondria, we observed an increase in both the mAβ42 and oAβ42. Therefore, we decided to assess if MERCS-related proteins are affected in cells incubated with mAβ42 and indeed Mfn2 levels were downregulated as shown in other models.

Since autophagosomes can be formed at MERCS we decided to investigate if the increase in MERCS had an effect on autophagosome formation. We started by evaluating basal autophagy in WT- and APP^{NL-F}-derived PCN, where we observed an increase in LC3B-II and a decrease in p62 protein levels, in the KI model suggesting up-regulation of autophagy. Thereafter, we starved both WT- and APP^{NL-F}-derived PCN and observed that autophagosomes were formed faster in the KI model than in WT. Interestingly, the number of MERCS per mitochondria was increased right before autophagosome formation in each model and Mfn2 and VDAC1 protein levels were altered. Therefore, we hypothesise that the increased connectivity between ER and mitochondria is needed for the formation of mature autophagosomes. ATP has been shown to be essential for autophagosome formation and thus we decided to assess mitochondrial function during the starvation

process. Indeed, we observed that starved WT PCN showed an increase in basal respiration, ATP production and ΔΨm at the same time as the number of MERCS per mitochondrian are increased.

This data suggests a new mechanism as to how Aβ affects cell homeostasis. We show that Aβ itself increases both the number of MERCS and number of MERCS per mitochondria, affecting mitochondrial function and autophagosome formation. Furthermore, we show, for the first time, that autophagosome formation during starvation leads to the increased proximity between ER and mitochondria and upregulation of mitochondria function. Further characterisation in PCN derived from APP^{NL-F} will be performed in order to understand how Aβ alters autophagosome formation as well as mitochondrial function and dynamics.

4.3 STUDY III: MITOFUSIN-2 KNOCKDOWN INCREASES ER-MITOCHONDRIA CONTACT AND DECREASES AMYLOID β-PEPTIDE PRODUCTION

Since we observed that MERCS are altered in AD and that Aβ is formed in MERCS we decided to evaluate if modulation of MERCS affected Aβ levels. In order to modulate MERCS, we decided to KD Mfn2 in HEK293 cells overexpressing APP^{Swe}. Due to the pivotal role of Mfn2 in cell homeostasis, we first assessed cell viability and we observed no differences in cytosolic reducing power or plasma membrane permeability upon KD of Mfn2. Since Mfn2 KD could affect mitochondrial dynamics and bioenergetics we also decided to assess ATP levels. No differences were detected in ATP levels upon acute KD of Mfn2. We believe this could be related to a compensation mechanism since, although not significant, we observed an increase in Mfn1 expression upon Mfn2 KD. However, upon ablation of Mfn2, we saw a significant increase in MERCS length and percentage of mitochondrial surface in contact with ER. This data was confirmed by the observation that ER-mitochondria Ca^{2+}-transfer was increased upon induction of Ca^{2+} release from ER using ATP and carbachol. Interestingly, the reduction of Mfn2 levels led to decrease of both intra- and extracellular levels of Aβ40 and Aβ42 coupled to impaired γ-secretase assembly and decreased activity (measured by the production of AICD). No changes were found in neprilysin activity (major Aβ degrading protein).

This data was surprising since we observed in **Study I** and **Study II** that an increase in Aβ is connected to an increase in MERCS. However, in this study, acute increase in MERCS leads to a decrease in Aβ levels. Several explanations could be considered: the fact that in the first studies we evaluated MERCS ultrastructure in

increased Aβ models, whereas in this study we modulated MERCS and evaluated Aβ; the fact that in the present study we performed an acute MERCS modulation while the models on **Study I** and **Study II** we measure MERCS in basal conditions; the model used in the present study is an immortalised cell lines derived from kidney cells while in the previous studies brain or primary cultures were used which have, as mentioned before, a substantially different mitochondrial metabolism. However, I believe that the observed discrepancy is connected to the inhibition of Aβ formation by MERCS **(Figure 8)**. Under normal conditions, Aβ is formed, among other places, in MERCS. When the levels of the peptide increase, this leads to an increase in the connectivity between ER and mitochondria (probably *via* Mfn2), which results in an inhibition of Aβ production, in a negative regulation feedback (similarly to what was observed in autophagosomes[220]), at least during the time frame studied. This mechanism would allow MERCS to regulate Aβ levels, lowering its levels when they are too high. Our data supports partly this model since all the models with increased Aβ we studied showed a decrease in Mfn2 levels and an increase in the number of MERCS. Furthermore, the increased number of MERCS observed in AD could be a rescue mechanism where the cells are trying to reduce the elevated levels of Aβ. Regardless of the exact unknown mechanism, our data reveals a new mechanism in which we show that modulation of MERCS alters Aβ formation. This new cellular pathway could be of great importance in understanding the mechanisms underlying the role of MERCS in AD.

4.4 STUDY IV: TOM70 SUSTAINS CELL BIOENERGETICS BY PROMOTING IP3R3 - MEDIATED ER TO MITOCHONDRIA Ca^{2+} TRANSFER

Previous findings showed that Aβ is imported into mitochondria via the translocase of the outer membrane (TOM)[247] and that Aβ is partially generated in MAM.[52] Therefore, we hypothesise whether TOM proteins are localised in MERCS and, if so, what their role is in this subcellular region. We started by showing that whilst TOM70 formed specific cluster along the OMM, TOM20 was ubiquitously covering most of the mitochondrial network. Curiously, TOM70 clusters were frequently co-localized with the ER, hinting that TOM70 is present in the OMM in proximity of MAM. In fact, we showed that both TOM70 and 20 are present in MAM-OMM-enriched subcellular fractions, that they are in close proximity with IP3R3 (as observed by PLA) and that they also co-precipitate with IP3R3 showing that these OMM proteins are not only close to MAM but also interact with MAM-related protein

IP3R3. Interestingly, we also found that upon knockdown of TOM70, but not of TOM20, there was a decrease in IP3-dependent Ca^{2+} release from ER to mitochondria and a decrease in Ca^{2+} levels inside mitochondria, suggesting a possible alteration and decrease in the contacts between ER and mitochondria. Surprisingly, when we assessed MERCS upon siRNA knockdown of TOM70 using both immunocytochemistry and TEM, no differences were found in MERCS. Curiously, since endogenous levels of IP3R3 where changed after TOM70 KD, we assessed IP3R3 distribution and we found less IP3R3 co-localizing with the OMM and less interaction between IP3R3-Grp75.

Altogether, this data suggests that TOM70 has a role in recruiting and/or stabilising IP3R3 localisation at MERCS but does not act as a scaffolding protein itself. To our knowledge, this is the first time that a protein has been shown to affect MERCS function without altering its ultrastructure. **(Figure 7)**. A decrease in mitochondrial Ca^{2+} leads to changes in mitochondrial respiration and TCA cycle, affecting ATP formation and consequently decrease of cell number and induction of autophagy. Currently, we do not know if TOM70 also has a role in the import of Aβ through the OMM. TOM70 is only transiently associated to the TOM complex therefore it is possible that the role of TOM70 in recruiting IP3R3 to MAM is independent from its function in the protein import machinery.

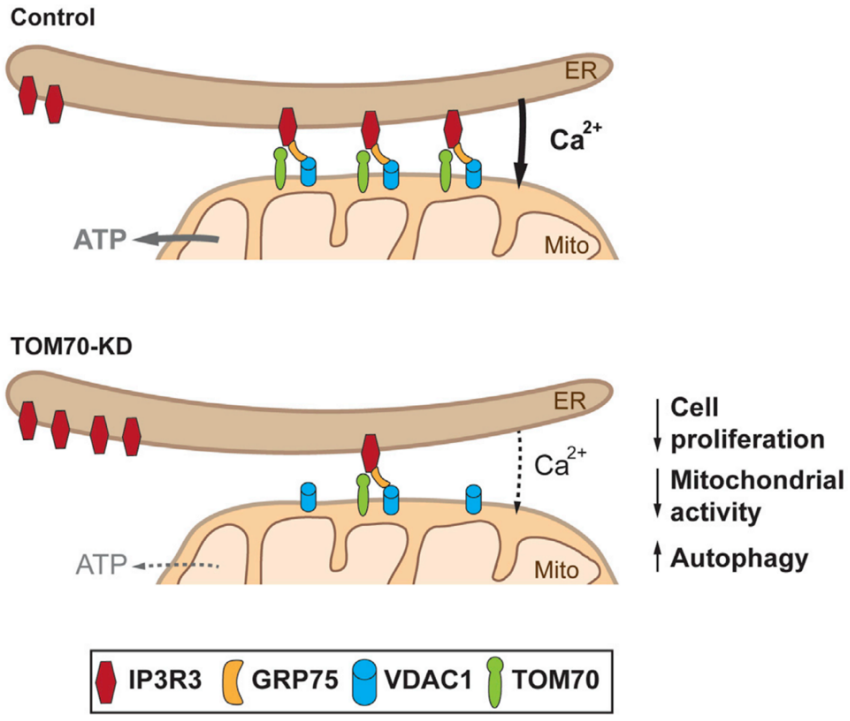

Figure. 7 Graphical Abstract of Study IV. KD of TOM70 leads to decreased in Ca^{2+} shuttling from the ER to mitochondria, cell proliferation, and mitochondrial activity, increase in autophagy and alteration in localisation of IP3R3 in MAM. (from Filadi et al. 2018, *Current Biology*)

5 CONCLUDING REMARKS AND FUTURE WORK

AD is the most common form of dementia in the world and, as of today, there are still drugs that can stop the progression of the disease. Several clinical trials to find a drug that stops the ongoing neurodegeneration process have failed. Therefore, researchers are currently focusing on developing multi-target therapies. The remaining issue is that several mechanisms and pathways connected to AD remain unknown. Therefore, there is a need to further understand the molecular mechanisms underlying the pathology, specifically the ones underlying the formation of the pathology hallmarks.

The aim of this thesis was to further understand the role of mitochondria-ER contact sites in AD. Even though MERCS were first described in 1990 it was not until 2009 that a connection between this subcellular region and AD was established. This allowed the connection of several of the biological processes altered in AD to one site within the cell (eg. altered glucose metabolism, Ca^{2+} homeostasis, autophagosome formation/maturation, phospholipid and lipid metabolism). Although, some new evidence has emerged over the years, we still do not know if alteration in MERCS is a cause or consequence of AD. Further,ore, how Aβ and MERCS regulate each other remains elusive. What we do know, together with previously published articles and the studies in this thesis, is that they are intrinsically connected. This thesis contributes to the field by showing that:

- during ageing there is an increase in the number of MERCS as well as in demented patients showing that MERCS dynamics change not only in pathology but also with the normal ageing;
- the number of MERCS positively correlated with the levels of ventricular Aβ42 and models with increased Aβ levels show an increment in MERCS, a decrease of Mfn2 protein levels and alterations in mitochondria function and autophagosome formation;
- as increase of MERCS (by Mfn2-KD) leads to a decrease of Aβ formation;
- it is possible to modulate Ca^{2+} activity in MERCS without changing its ultrastructure by modulating TOM70 protein levels.

Even though this thesis could not answer whether the increased in MERCS is a cause or a consequence of the unbalanced Aβ formation in AD, it certainly helped to understand better of the mechanisms underlying. Based on the data above we believe that MERCS have a pivotal role in AD and cell homeostasis. In fact, levels of MERCS are increased in different AD models with increased Aβ levels. In addition, inhibition of oAβ with scFvA13 rescue the numbers of MERCS similarly to the control showing that oAβ itself modulates MERCS. Consistently, the levels of the MERCS negative regulator Mfn2 were decreased in all the Aβ models analysed, which could explain the observed increment of MERCS. In fact, Mfn2 knock-down led to an increase of MERCS length and reduction in the levels of Aβ. Therefore, we hypothesise a new model where Aβ affects MERCS *via* Mfn2 although we were unable to identify the exact mechanism **(Figure 8)**. Although this data seems contradictory at the first sight, we believe that this oppsoing data is a result of a a negative feedback mechanism from MERCS to Aβ. Our hypothesis is that Aβ is formed, among other places, in MERCS. However, at high concentration, Aβ induced an increase in the connectivity between ER and mitochondria (as observed in **Study II**). This increment in MERCS leads to the ceasing of Aβ formation either through modulation of APP processing and γ-secretase assembly/activity in MERCS (as observed in **Study III**) and/or its degradation/secretion via exosome/autophagosomes (**Figure 8)**. Additionally, since Aβ leads to general cell failure, the augmentation of MERCS could be a cellular rescue mechanism since it would try to re-establish normal cell metabolism and biological functions. However, prolonged increase of MERCS (as a result of to constant high levels of Aβ) can ultimately result into cell death (eg. apoptosis) due to subsequent mitochondrial Ca^{2+} overload and alteration in autophagosome formation/maturation.

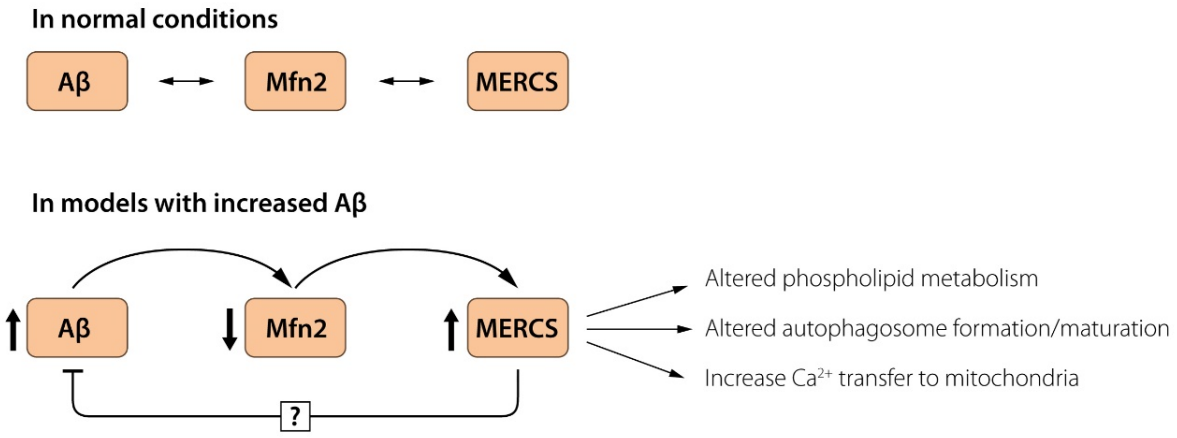

Figure. 8 Under normal conditions there is a balance between Aβ, Mfn2 levels and MERCS. In pathological conditions, an increase in Aβ, decrease in Mfn2 and an increase of number of MERCS is observed. How MERCS regulate Aβ levels is not known but we believe that a negative feedback loop could be involved in this process

Therefore, this thesis contributes to a better understanding of how MERCS regulates and is regulated in AD. Although, since the field of MCS and MERCS is still in its early days, I believe that, before we clearly understand the mechanisms behind MERCS in AD pathology, further progress will require a better understanding in the basic knowledge of MERCS: Are there different types of MERCS? If yes, do these different types have different tethering proteins? If so which ones are affected in AD? Do they have different functions? Does the MAM have a different composition? Does the composition of MAM change during different metabolic conditions? What are the signals that promote the proximity between ER and mitochondria? After we understand these basics and the consequences of MERCS modulation, we can then start thinking about MERCS as a possible target for drug treatment.

6 ACKNOWLEDGEMENTS

Firstly, I would like to thank everyone that has contributed to the birth of this thesis.

To **Maria Ankarcrona**, my main supervisor. When I started in your lab I was so inexperienced and now, after five and a half years, I feel like I have grown so much, both as a person and as a researcher. It was a joy to also see you growing as a researcher and also conquering important goals. I have appreciated the freedom you have given me in the lab; it has not always been positive and has cost me some nights of sleep, but in the end it helped me to become a more independent researcher.

To my co-supervisors:

Helena Karlström for all your support through the years and for always being available when I need you. Even though you are an independent group leader it always felt like we were working as one group, with different research interests.

Bengt Winblad for all your work over the years, even before I arrived. Thank you for providing, together with **Gunilla Johansson**, the great environment we have in our division. Without you our division would not be what it is today.

Per Nilsson for all the support in the autophagy field. Your inputs were highly appreciated. A big thank you for the APP^{NL-F} and APP^{NL-G-F} mice! Without those, Study II would not have been possible. Ah, and thank you for always telling me that I need to work longer and harder: “Are you leaving already? Why are you not working?” Per, you are back in Sweden, people are allowed to leave before 22:00!

To our group and the Portuguese Mafia I would like to say a big THANK YOU:

To **Bernadette “Bernie” Schreiner**, my lab goddess. I owe you almost every skill I have developed in the lab, as well as my scientific thinking. I really appreciate all the time you spent with me and all the teachings, in and out of the lab. Your twisted mind certainly made me a better and more open minded person; I hope I will not start sleeping with a bottle of wine next to me! Ich mag dich!

To **Birgitta “Bitti” Wiehager**, for all the cell lab teachings and for always taking care of us, our cell lab and our cells. I think about you every Wednesday and Thursday. Please come back!

To **Catarina “Cata” Pinho**, for all the help through the last seven years, as a friend and as a colleague. Also, for being with me since day 1 in Stockholm, literally. I appreciate every moment with you and **Diogo** and the fact that you guys brought me along when I did not know anyone. Stockholm was way brighter and funnier with

you two. Tell **Clarinha** to always nourish and take care of Nuninho! To the **little one**, cannot wait to meet him/her!

To **Giacomo "too many nicknames to put here" Dentoni**, where to start? Pay attention because I will only say it once but you will hear it twice, okurr? Thank you for the FRUITFUL discussions, for throwing pillows at me when I snore and bullying me, for drawing me in African dresses, for sharing PhD projects, for all the laughs and all stupid things, for always retaliating with sassy comments, for the Miami accent, Google translator, "heavens above", "club 96", amigas Latinas and so many other memories. This thesis would not have been possible without you, at all. You have helped me a lot, during the times you were around. Thank you. Ah, another thing: Jesus didn't die for acknowledgments did he?

To **Daniel "Dani" Oliveira**, the CADASIL member of our group. Never judge a book by it is cover, right? I am very glad you joined Helena's group and shared the office with us. I certainly became "darker" and you certainty became more "colourful", until you went downhill again ranhoso. I never thought I would have to give "Britney Spears' therapy" to a 38 year old metal head man!

To **Luana "the pH OCD (she doesn't like Lulu)" Naia** for bringing a fresh breeze into the lab, with a lot of knowledge about mitochondria. Your critical point of view made me start questioning things that I have not before. I will definitely bring this with me. Ah, and I will always check the pH of my solution!

To **Filipa "Minion" Viegas** for being such a good and efficient student (although I am not sure if your MEF cells would agree with me). You helped me recover from a "fruit salad" traumatic experience, believe me. Thank you for always being in a good mood and for reminding me of myself when I was a master student. Keep it up and don't let that joy disappear.

I would also like to thank everyone in our division for all the good times, scientific input and amazing social environment: **Chenhong** for all the food adventures and "out-of-the-box" conversation we had. Thank you for all the romantic moments we had in Sweden and in Portugal. I will never forget our night in Cais do Sodré drinking Ginginha; **Joana** I am so glad you don't own a flip phone anymore and we can actually write to each other like it's the XXI century. Thank you for being such a "desbocada", it triggered so make laughs; **Laetitia** for all the conversations in the corridors and in the kitchen back at NOVUM and, of course, for being my petit déjeuner; **Kirsten** for all the inputs and company in the office and in the lab; **Hazal** for providing the circumstances that created a new scar on my toe and allowing me

to add Turkish dancing skills to my CV; **Ceren** for being my favourite Instagram celebrity; **Konstantinos** for being such an enthusiastic nerd and for all the laughs we have had; **Jolanta** for teaching me to never judge a book by its cover. We are so different but I loved to be around you; **Axel** for saying inappropriate things and do not keep a secret, **Medoune** for bringing the cool spiders to the lab; **Maria Leal**, my Spanish cousin; **Julen** “spale my rife”, “you dlive me clazy”; **Emilia** for teaching me how to properly cook broccoli; **Raul** for always being in a contagiously good mood; to **Shao-Bo** for bringing delicious treats from China and other labs; to the Portuguese **Erika B.;** and to the other two members from the Söderåkra gang that have left: **Carlos** and **Helen**. We all had amazing times together and I will truly miss you guys.

I would also like to thank **Patricia**, **Heela**, **Ipsit**, **Lorena**, **Camila**, **Cris**, **Antonio**, **Christa**, **Evelyn**, **Silvia**, **Homira**, **Pavel**, **Lars**, **Susanne**, **Sophia**, **Erik**, **Marianne**, **Angel**, **Janne**, **Mia**, **Elisabet**, **Eric** and our “old neighbours”, the division of Clinical Geriatrics. To **Eva Kallstenius**, thank you so much for all your fast and efficient help. I appreciate how you were always so helpful, nice and available. I am very glad we became “neighbours”!

To the doctoral council of NVS (**Sophie**, **Emelie**, **Anna**, **Mats**, **Carrie**, **Pavla**, **Emerald**, **Gustav**) that I had the chance to be part of. It was good practise to get to know the organizational structure of KI and to help improve the work environment of PhD students in our department.

To **Agustin “Agus” Sola Carvajal** a big thank you for your wise words whenever I needed them. You are a good friend and an amazing researcher. Also, thank you for giving me shelter when I become homeless. I cannot wait for Fredagsmys and gossip times together!

Since research is never done individually, I would also like to thank all of our collaborators in and out of Sweden: To **Paola Pizzo** and **Riccardo Filadi** for all the interesting discussions and for the teachings about Ca2+; to the electron microscopy unit (EMil): **Kjell Hultenby**, **Lars Haag**, **Eva Idsun Jonsson** and **Eva Blomèn** for all the help with electron microscopy. Without you, none of the publications in this thesis would have been possible. I am very thankful; to **Ville Leinonen**, **Tuomas Rauramaa** and the rest of the Finnish collaborators for the human biopsies. To **Hamasaki-san** for always challenging me and pushing me out of my comfort zone. Every time we had the chance to discuss my project you always contributed a lot. Part of this thesis would not have been possible without you. Also, thank you for all the food tips! おいしい！ The places we went together for food were amazing. I

would kill for some しゃぶしゃぶ right now. I miss Japanese food. To **Yoshimori-sensei**, thank you for the opportunity to visit your lab. Working in Japan certainly changed my life. **大阪**と**日本**はすごいでした。ありがとうございます。

Obrigado aos “não-cientistas” da minha vida que podem não ter contribuido directamente para esta tese mas que me criaram, cuidaram de mim e me incentivaram a nunca desistir. Sem eles nunca teria alcançado nada desta vida, incluido esta tese.

Aos meus **pais**. Se calhar não vos digo o suficiente mas obrigado por tudo, amo-vos. Sem vocês eu não seria ninguém. Obrigado pelos sacrifícios que foram feitos tantos nos últimos tempos para que esta tese fosse possível comos nos últimos 27 anos. Um dia será reciprocado. Esta tese é dedicada a vocês.

To **Joe**. Thank you for always being there, make me laugh and happy. I appreciated it during all this time. Without you this journey would not have been as interesting. Cannot wait for the next adventures and I hope it includes piggy dances, singing, Wing, Aliana Glande and food, a lot of food. “Shall we? Let’s go!”

À minha **‘bó Ana** por entre outras coisas, me teres acolhido quando decidi ir estudar para o Porto e nunca pediste nada em troca. À minha **‘bó Guida** pelas canjas depois de 6h horas de viagem. Lamento que não tenhamos sido mais rápidos com a nossa investigação. Prometo-te que um dia ninguém terá de passar pelo sofrimento que estás a passar. Aos meus avôs, **‘bô Quim** e **‘bô Tone**, tenhos saudades vossas. Ao meus tios (**Beta, Beto, Paula, Pedro, Guida, Mino, Cinda** e **Luís**) e primos (**Beatriz, Mário, Ju, Diana, Hugo, Luís Miguel** e **Silvana**). À minha família Algarvia: **Lena, Sebastião, Rodrigo e Miguel** que sempre me trataram como se fosse um deles. Obrigado por todos momentos e por estarem sempre lá. Gosto muito de vocês! To **Fábio, Silvia, Melina, Carlos, Mafalda, Tomas** and **Andreas.** Aos meus professores **Maria Paula Abreu**, **Sérgio Leal** e **Ione Dionísio** por terem dispultado o meu gosto pelas ciências naturais.

I would like to thank Japanese Society for the Promotion of Science, Stiftelsen Gun and Bertil Stohnes, Svenska Läkaresällskapet, Stiftelsen för Gamla Tjänarinnor and other foundation for funding the projects that are part of this thesis.

Special thank you to everyone that helped writing and proof-read this thesis and to Mattias Karlén for the majority of the figures in this thesis.

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